REGULATION OF THE EXPRESSION OF BDNF AND ITS RECEPTORS IN THE DEVELOPING NERVOUS SYSTEM

Michelle Yvonne Robinson

A Thesis Submitted for the Degree of PhD
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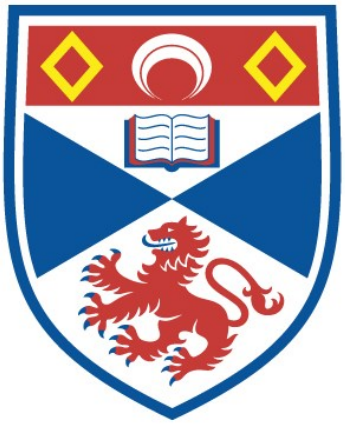
University of St Andrews



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Submitted for the Degree of Ph.D.

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Contents

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Declarations

I, Michelle Yvonne Robinson, hereby notify that this thesis, which is approximately 50,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application fo a higher degree.

Date 10/4/96 Signature of candidate

I was admitted as a research student at St. George's Hospital Medical School in June, 1993, and as a candidate for the degree of Ph.D. at the University of St. Andrews in November, 1993; the higher study for which this is a record was carried out in St. George's Hospital Medical School and the University of St. Andrews between 1991 and 1996.

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List of Figures

Figure 13, page 139

Graph of the level of full-length *trk*B mRNA relative to L27 mRNA (fg *trk*B mRNA per pg L27 mRNA) in the vestibular and nodose ganglia of chicken embryos at stages 17 to 24.

Figure 14, page 141

Graph of the level of p75 mRNA relative to L27 mRNA (fg p75 mRNA per pg L27 mRNA) in the vestibular and nodose ganglia of chicken embryos at stages 17 to 24 (stage 20 was the earliest stage at which nodose ganglia were studied).

Figure 15, page 142

Graph of the level of BDNF mRNA relative to L27 mRNA (fg BDNF mRNA per ng L27 mRNA) in the heart, hindbrain, and otic vesicle of chicken embryos at stages 16 to 24.

Figure 16, page 144

Graph of the level of full-length *trk*B mRNA (fg per neuron) at 12 hourly intervals in low-density cultures of stage 19 chicken nodose neurons grown without or with BDNF in the culture medium for 72 hours.

Abbreviations

a/b FGF Acidic/basic fibroblast growth factor

BDNF Brain-derived neurotrophic factor

ChAT Choline acetyl transferase

CIAP Calf intestinal alkaline phosphatase

CMF-PBS Calcium- and magnesium-free phosphate-buffered isotonic saline

CNTF Ciliary neurotrophic factor

ddNTP Dideoxynucleotide triphosphate

DEPC Diethylpyrocarbonate

DiI 1', 1'-Dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate

DG Diacylglycerol

DMSO Dimethyl sulphoxide

DMTG Dorsomedial trigeminal ganglion

DNase 1 Deoxyribonuclease 1

dNTP Deoxynucleotide triphosphate

DRG Dorsal root ganglion

DTT Dithiothreitol

E Embryonic day

EDTA Ethylenediaminetetraacetic acid

Erk 1, 2 Extracellular signal-regulated kinase 1, 2

FGF(-5) Fibroblast growth factor(-5)

GABA γ-aminobutyric acid

GAP Ras GTP-ase activating protein

GDNF Glial cell line-derived neurotrophic factor

HIFCS Heat-inactivated foetal calf serum

HIHS Heat-inactivated horse serum

IMS Industrial methylated spirits

IL-6 Interleukin-6

IP_3 Inositol trisphosphate

IPTG Isopropyl-β-D-thiogalactopyranoside

L broth/agar Luria broth/agar

LIF Leukaemia inhibitory factor

MAPK Mitogen-activated protein kinase

MAPKK Mitogen-activated protein kinase kinase

MOPS 4-Morpholinepropanesulfonic acid

NGF Nerve growth factor

NPY Neuropeptide Y

NT-3, -4/5, -6 Neurotrophic factor-3, -4/5, -6

OD Optical density

PEG Polyethylene glycol

PI-3 kinase Phosphatidylinositol-3 kinase

PLC-γ1 Phospholipase C-γ1

P-ORN Poly-DL-ornithine

RA Retinoic acid

RNase Ribonuclease

RT-PCR Reverse transcription-polymerase chain reaction

SCF Stem cell factor

SDS Sodium dodecyl sulphate

SH2 Src homology 2

SNT *suc*-associated neurotrophic-factor-induced tyrosinephosphorylated target

SOS Son of sevenless

SCG Superior cervical sympathetic ganglion

TAE buffer Tris-acetate-EDTA buffer

TBE buffer Tris-boric acid-EDTA buffer

TGF-β (1, 2, 3) Transforming growth factor-β(1, 2, 3)

TEMED N, N, N', N'-Tetramethylethylenediamine

TH Tyrosine hydroxylase

TMN Trigeminal mesencephalic nucleus

VLTG Ventrolateral trigeminal

VRC Vanadyl-ribonucleoside complex

X-gal 5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside

Abstract

BDNF binds to two transmembrane receptors: *trk*B, which is a tyrosine kinase essential for signalling, and p75, which is a common neurotrophin receptor whose role is contoversial. To determine the relationship between BDNF synthesis, BDNF receptor expression, and neuronal responsiveness, the expression of BDNF, *trk*B, and p75 mRNAs were studied for different populations of sensory neurons whose axons reach their targets and become dependent on BDNF for survival at different times. BDNF mRNA was expressed in the peripheral and central targets of these neurons prior to the arrival of sensory axons. The onset of BDNF responsiveness was preceded by the expression of first p75 mRNA then *trk*B mRNA, and neurons that start responding to BDNF early were the first to express *trk*B mRNA. BDNF upregulated *trk*B mRNA expression just shortly before the onset of BDNF dependence.

BDNF is expressed not only in sensory neuron targets but in some of these neurons themselves. To determine whether BDNF is synthesized in NGF-dependent or BDNF-dependent neurons, BDNF mRNA expression was studied in purified populations of cranial sensory neurons that depend on either NGF or BDNF for survival. During the period of neuronal death, BDNF mRNA expression was highest in NGF-dependent cutaneous sensory neurons, lower in BDNF-dependent cutaneous sensory neurons, and undetectable in BDNF-dependent proprioceptive neurons. In co-culture, NGF-dependent neurons promoted the survival of BDNF-dependent neurons by the production and release of BDNF, implying a paracrine role for BDNF during the period of naturally occurring neuronal death.

To determine if the level of p75 expression in sensory neurons is related to the particular neurotrophin they require for survival, p75 mRNA levels were measured in purified populations of cranial sensory neurons. No clear relationship between the level of p75 mRNA expression and neuron type was observed. Studies of the regulation of p75 mRNA expression in sympathetic neuroblasts revealed that retinoic acid increased and membrane depolarization using KCl decreased the levels of p75 mRNA.

CHAPTER 1

General Introduction

Early Neural Development

The following is a brief account of the events occurring in early neural development. These are described more fully in a review by Schoenwolf and Smith (1990). The onset of neural development is marked by the formation of a dorsally-positioned groove along the ectodermal surface of the gastrula. The neural groove then widens, accompanied by the thickening of the ectoderm lateral to it which forms the neural plate on the dorsal side of the embryo. The margin of the neural plate bears ridges called neural folds which start fusing near the anterior end to form the neural tube. Most of the cells from which the brain and spinal cord are formed come from the neural tube. The beginning of brain development is marked by a slight anterior swelling of the neural tube.

During neural tube closure, a group of cells, the neural crest, detach from the margin of the neural ectoderm and form the peripheral nervous system. They come to lie in a band along the length of the neural plate and finally position themselves dorsally and laterally to the closing neural tube. The cells then undergo a period of migration into the periphery followed by differentiation into spinal and autonomic ganglia, glial cells of the autonomic and sensory nervous system, and other non-neuronal tissues such as melanocytes, chromaffin cells of the adrenal medulla, cartilage, blood-forming cells, connective tissue covering the brain and spinal cord, and parts of the facial bones.

Neural tissues are also formed from ectodermal placodes which appear as thickenings of the ectoderm in the head region. Structures such as the anterior pituitary gland, the olfactory sensory epithelium, and the lens are derived from placodes. The more caudally postioned 'neurogenic' placodes give rise to epithelial cells that migrate to form the neurons of certain cranial sensory ganglia.

The Neurotrophic Theory

During development, neurons extend axons towards their target fields to establish contacts with target cells and make appropriate synaptic connections. Since more neurons are generated than required, a mechanism must exist for controlling the number that finally make successful synaptic connections in the target field. This process is marked by a distinct period of neuronal cell death, which commences shortly after the first neurons innervate their targets (Cowan *et al.*, 1984). It has been assumed that the target field itself controls the size of neuronal populations that innervate it by synthesizing a limiting supply of a neurotrophic factor on which the neurons are dependent for survival. This has been embodied in the neurotrophic theory (Davies, 1988a; Barde, 1989; Oppenheim, 1991; Thoenen, 1991).

Most of the studies supporting the theory have been on nerve growth factor (NGF), the first neurotrophic molecule to be identified and completely characterized. It was shown that NGF is produced by cells in the target field of neurons that require it and that its availability regulates neuronal survival. NGF is bound by the neuron and the NGF-receptor complex internalized and transported to the cell soma (Levi-Montalcini, 1987). It has also been demonstrated that NGF is produced in the target field in proportion to the density of innervating neurons (Heumann *et al.*, 1994; Shelton and Reichardt, 1984), especially during development (Harper and Davies, 1990).

An alternative proposal to limited production of neurotrophic factor is one of limited access to it. Enough neurotrophic factor would be produced to support all the developing neurons and whether or not a neuron survives would be governed by its ability to form sufficient neuronal branches and synaptic sites through which to obtain the factor (Oppenheim, 1989). Studies in support of this theory make use of hyper-innervated embryonic chicken muscle produced by pharmacological paralysis. It has been demonstrated that extract from this muscle promotes motoneuron survival with a similar efficiency to that from normal embryos both *in vitro* (Tanaka, 1987) and *in vivo* (Houenou *et al.*, 1989), which suggests that neurotrophic factor is synthesized at similar levels in normal and hyper-innervated muscle.

However, further studies on the nervous system and other NGF-related neurotrophic molecules have shown that the control of target innervation is not so simplistic. The members of the NGF family of neurotrophic factors (or neurotrophins) are brain-derived neurotrophic factor (BDNF), neurotrophin-3, -4/5, and -6 (NT-3, -4/5, and -6). Both NGF, its related neurotrophic molecules, and their receptors will be discussed in more detail in later sections of this Chapter.

Recent studies have exposed the limitations of the neurotrophic theory. Apart from obtaining neurotrophic support from innervated target cells (the classical retrograde mechanism) (Levi-Montalcini, 1987; Thoenen *et al.*, 1987), neurons might also obtain it from other neurons which are afferent (an anterograde mechanism). Oppenheim (1991) has described how a number of neuronal types degenerate when afferent input is lesioned, providing strong evidence for an anterograde mechanism. Neurotransmitter molecules, which are transported anterogradely, may also influence neuronal survival. Lipton and Kater (1989) have documented the effects on neuronal survival and differentiation of a variety of neurotransmitters.

A further source of neurotrophic support may come from cells ensheathing nerves. Heumann *et al.* (1987), have demonstrated that a high rate of NGF synthesis is maintained by peripheral nerve-ensheathing cells during development which decreases in adulthood. It also increases briefly when the nerve is lesioned. This suggests that the nerve cells receive a local supply of NGF prior to target innervation during development or before re-contacting target organs during regeneration.

Some types of neuron have been found to synthesize both neurotrophic factors and their respective receptors and so respond to their own neurotrophic factors in an autocrine mechanism. Dorsal root ganglion (DRG) neurons, sympathetic neurons, and hippocampal pyramidal neurons synthesize both BDNF and its receptor, *trk*B (Ernfors *et al.*, 1990a; Klein *et al.*, 1990b; Wetmore, *et al.*, 1991; Schecterson and Bothwell, 1992; Wright *et al.*, 1992). Direct evidence for autocrine support by BDNF has come from studies using both early (Wright *et al.*, 1992) and adult (Acheson *et al.*, 1995) DRG neurons. Neurotrophic factor secreted by one neuron may also be available to

other neurons within a population, possibly via a paracrine mechanism of neuronal support. Autocrine or paracrine support mechanisms would obviously provide a function distinct from that of neuron-target interaction. They may play a role in maintaining neurons prior to target field innervation (Korsching, 1993).

The Structurally-Related Family of Neurotrophic Factors

The work of Hamburger and his colleagues provided evidence that neurons require more than nutrients in order to survive. Their experiments involved the removal of a wing or limb bud in the chick embryo prior to innervation, with resultant death of the innervating sensory, autonomic, and motor neurons. These results suggested that the peripheral tissues might produce certain substances essential for the maintenance of neurons (review by Levi-Montalcini, 1987). Experiments directed at understanding the nature of this peripheral influence led to the discovery of the first neurotrophic factor, NGF, in the early 1950's. Levi-Montalcini and Hamburger (1951, 1953) observed that transplantation of a mouse sarcoma into chick embryos after limb bud removal resulted in the enlargement of sympathetic and dorsal root ganglia and hyper-innervation of the tumour. Studies of the substance released by the sarcoma were initially carried out using neurite outgrowth assay techniques for chick sympathetic and dorsal root ganglia (Levi-Montalcini *et al.*, 1954) and ultimately led to the purification of NGF.

Other members of the family of structurally-related polypeptide neurotrophic factors (i.e., BDNF, NT-3, NT-4/5, and NT-6) have been identified more recently. Elucidation of the primary structures of these molecules has revealed that their basic conformations are determined by strictly conserved cysteine-rich domains. Distinct regions of variation between the molecules are responsible for their binding to specific high-affinity receptors, located on a variety of neuronal types, through which their biological actions are mediated. The neurotrophic factor-receptor interaction also involves a low-affinity receptor component which is common to all the factors. The spatial and temporal expression of the neurotrophic factors and their receptors during development plays an essential role in regulating the formation of the nervous system.

Nerve Growth Factor (NGF)

As the first neurotrophic factor to be isolated, NGF remains the best characterized with respect to structure and function. It promotes the survival of sympathetic neurons (Chun and Patterson, 1977; Greene, 1977), certain types of sensory neurons (Davies and Lindsay, 1985), and basal forebrain cholinergic neurons (Hatanaka *et al.*, 1988).

The NGF protein was purified from the submandibular gland of the male mouse (Angeletti and Bradshaw, 1971; Angeletti *et al.*, 1973), where it is found in great quantities. Why NGF is located here in such quantity (approximately 0.1% of total protein; Cohen *et al.*, 1960) is uncertain. Aloe *et al.* (1986) suggest that it might be important in the aggressive behaviour of male mice as the salivary glands release large quantities of NGF into the bloodstream during fighting. This, however, is not found in other aggressive animals.

Significant levels of NGF are also found in other exocrine tissues and their secretions. These include snake venom (Bailey *et al.* 1975), guinea pig prostate (Chapman *et al.*, 1981), bull semen and seminal vesicles (Hoffman and Unsicker, 1982), and the submandibular gland of the African rat *Mastomys natalensis* (Aloe *et al.*, 1981). No explanation has been found for the presence of NGF in these regions; there appears to be no influence on the developing or mature nervous system (Thoenen and Barde, 1980; Harper and Thoenen, 1981). Most studies have been carried out using mouse NGF because the submandibular gland of male mice is such a rich source of the factor.

Protein Structure

NGF isolated from the submandibular gland of the male mouse has a molecular weight of 130,000 Da. It is synthesized as a complex of three subunits, α , β, and γ. Only the β subunit has an effect on neurons. Hence, the active form of NGF is often referred to as β-NGF. It is generated from a 305 amino acid prepro-NGF by proteolytic cleavage and comprises a 26,000 Da dimer of two identical 118-amino acid monomers,

each containing three intrachain disulphide bonds (Bradshaw *et al.*, 1993). Loss of these bonds results in loss of biological activity (Greene and Shooter, 1980; Thoenen and Barde, 1980).

X-ray diffraction studies have recently been used to determine the three-dimensional structure of the mature molecule (McDonald *et al.*, 1991). Dimer formation occurs through hydrophobic contacts between the flat surfaces of each monomer. The hydrophobic core of each monomer bears the majority of amino acids common to all the neurotrophic factors, including the disulphide bonds. Several amino acids that are conserved in the neurotrophic factors contribute to NGF structure by forming hydrogen bonds (review by Ebendal, 1992). A group of positively-charged amino acids has been shown to be important in the interaction of NGF with the low-affinity receptor (Ibáñez *et al.*, 1992). Of the regions that differ between the neurotrophic factors, one forms three closely-associated β-hairpin loops in the NGF protein. It is possible that these regions are responsible for the different specificites each factor has for its high-affinity receptor (review by Ebendal, 1992).

Gene Structure

The mouse NGF gene is 45 kb in length and consists of five exons separated by four introns. The mature protein is translated from the transcript of a single 3' exon. The remaining 5' exons are smaller and undergo alternative splicing to produce at least four transcripts that encode precursor proteins that differ in their amino termini (Selby *et al.*, 1987). The two major transcripts give rise to precursor proteins of molecular weights 34,000 Da and 27,000 Da. The 34,000 Da precursor has a hydrophobic signal peptide 70 residues downstream from the initiation methionine, while the 27,000 Da precursor has a hydrophobic signal peptide at the amino terminus. Both are further processed through cleavage and glycosylation to give mature, biologically active protein (Edwards *et al.*, 1988). The reason why several different transcripts are produced is uncertain. Of the two major transcripts, the one encoding the larger precursor is the most abundant in mouse submandibular gland and in placenta from several species,

while the other transcript is predominant in all other tissues.

Brain-Derived Neurotrophic Factor (BDNF)

BDNF was the next neurotrophic factor to be characterized. It promotes the survival of sensory neurons that are unresponsive to NGF (Davies *et al.*, 1986a; Lindsay *et al.*, 1985), the dopaminergic neurons of the substantia nigra (Hyman *et al.*, 1991), and developing motoneurons under certain experimental conditions (Oppenheim *et al.*, 1992).

Molecular Structure

BDNF was first isolated from pig brain (Barde *et al.*, 1982). It was found to be a 12.3-kDa basic protein. Molecular cloning (Leibrock *et al.*, 1989) has shown that the BDNF protein has approximately 50% amino acid identity with NGF.

BDNF gene sequence information has been determined for a number of other species, including human and rat (Maisonpierre *et al.*, 1991) and chicken (Maisonpierre *et al.*, 1992). The rat BDNF gene has been shown to span over 40 kb of genomic DNA and consists of four short 5' exons and one 3' exon encoding the mature BDNF protein (Timmusk *et al.*, 1993). Eight different BDNF mRNAs with four 5' ends and two alternative polyadenylation sites are transcribed from this gene. BDNF mRNAs containing the first three 5' exons are expressed predominantly in the brain, whereas transcripts containing the fourth exon are found predominantly in the lung and heart.

Neurotrophin-3 (NT-3)

NT-3 was the third member of the NGF gene family to be identified and, in contrast to NGF and BDNF, this did not require prior purification of the NT-3 protein. Primarily, the research groups involved made use of the polymerase chain reaction (PCR) technique and the nucleotide sequence homology between NGF and BDNF (Hohn *et al.*, 1990; Maisonpierre *et al.*,1990; Rosenthal *et al.*, 1990).

NT-3 supports proprioceptive sensory neurons that project to skeletal muscle,

enteroceptive sensory neurons (Hohn *et al.*, 1990), and embryonic motoneurons *in vitro* (Henderson *et al.*, 1993).

Neurotrophin-4/5 (NT-4/5)

Hallböök *et al.* (1991) took advantage of the regions of conservation between NGF, BDNF, and NT-3 in a PCR-based search for further members of the NGF gene family. As a result, NT-4 was identified and sequenced for the clawed toad *Xenopus*. Mammalian versions of NT-4 have since been isolated from the rat and human genomes (Ip *et al.*, 1992). Ip *et al.* (1992) have also isolated a pseudo-NT-4 gene containing several frame shifts, an internal stop codon, only four of the six essential cysteine residues, and no cleavage site to process the mature neurotrophic factor. The biological activity of recombinant *Xenopus* NT-4 is similar to that of BDNF in that it promotes the *in vitro* survival of sensory neurons (Hallböök *et al.*, 1991). NT-4 has also been shown to support the survival of embryonic rat motoneurons *in vitro* (Henderson *et al.*, 1993).

Berkemeier *et al.* (1991) also used a PCR strategy to isolate DNA fragments encoding a protein they referred to as neurotrophin-5 (NT-5). This protein has the same sequence as that described for human NT-4 by Ip *et al.* (1992). Berkemeier *et al.* (1991) claim that NT-5 is a distinct neurotrophic factor from NT-4 as it is expressed at low levels in a few peripheral organs and can stimulate sympathetic neurons when in the form of a recombinant protein; properties not exhibited by *Xenopus* NT-4. However, because the sequences of NT-5 and human NT-4 are the same, mammalian NT-4 is referred to as NT-4/5.

Neurotrophin-6 (NT-6)

NT-6 was cloned from a genomic library of the teleost fish *Xiphorus maculatis* (Götz *et al.*, 1994) and remains the most recently identified member of the NGF family of neurotrophic factors. It bears the structural features characteristic of this gene family, including the six conserved cysteine residues and has been found to promote the

survival of a similar range of neurons to NGF.

Neurotrophic Factor Expression

in the Central Nervous System

NGF, BDNF, and NT-3 appear to be synthesized primarily in specific neurons in the brain and, therefore, target-derived influences are achieved through interactions between neurons (Ayer-LeLievre *et al.*, 1988; Gall and Isackson, 1989; Ernfors *et al.*, 1990a,b; Philips *et al.*, 1990; Isackson *et al.*, 1991). Several studies have demonstrated the changes in NGF mRNA and protein levels in various regions of the rat brain during embryonic and early postnatal development (Large *et al.*, 1986; Lu *et al.*, 1989; Mobley *et al.*, 1989). NGF mRNA and protein levels are initially low and uniform throughout the brain and do not reach a maximum until postnatal day 21 in the neocortex and hippocampus. During development, BDNF is expressed initially at low levels but increases to become the most widespread neurotrophic factor in different areas of the brain. It reaches its peak level of expression two weeks after birth. NT-3 mRNA is weakly expressed in the hippocampus and cerebellum and reaches its maximum level of expression shortly after birth. NGF, BDNF, NT-3, and NT-4 are expressed in the adult brain (Persson and Ernfors, 1992). NT-6 expression has been detected in a small region of teleost cerebellum beneath the midbrain tectum during organogenesis, in 8-day old fish, and in the adult (Götz *et al.*, 1994).

in Peripheral Tissues

NGF expression has been studied extensively in the peripheral whisker pad target field of the mouse trigeminal system. Davies *et al.* (1987) have demonstrated that NGF mRNA and protein are synthesized in the non-neuronal epithelial and mesenchyme cells of the target field at the time of arrival of the first nerve fibres. NGF, BDNF, and especially NT-3 are expressed in many peripheral target tissues such as skeletal muscle, liver, and gut. NT-3 is expressed predominantly in these peripheral tissues, being only weakly expressed in the brain, and is known to support the

proprioceptive neurons projecting to skeletal muscle and the somatosensory fibres of the nodose ganglion (Thoenen, 1991). NT-6 expression has been detected in adult teleost gill, liver, and eye with weak expression in skin, spleen, heart, and skeletal muscle (Götz *et al.*, 1994).

Neurotrophic Factor Receptors

The use of highly purified ^{125}I NGF in binding and cross-linking studies has facilitated the characterization of NGF receptors in the late 1970's (Sutter *et al.*, 1979; Schechter and Bothwell, 1981). It is now well established that members of the NGF family of neurotrophic factors recognize two types of receptor, distinguishable by their pharmacological properties (review by Meakin and Shooter, 1992). One type of receptor binds all the factors with low affinity and has a dissociation constant of 10^{-9}M. It is a cell surface protein with a molecular weight of 75 kDa in the human, although the rat protein is slightly larger, and is referred to as p75 for all species. The p75-neurotrophic factor moiety is not internalized. The precise role of p75 in mediating the biological effects of neurotrophic factors is not certain but it may form part of a complex or interact in some other way with the second class of receptors. These are products of the *trk* oncogenes, a family of related tyrosine kinase-bearing receptors. They show specific high affinity binding for the neurotrophic factors (the dissociation constants being in the low picomolar range for NGF and NT-3 but undetermined for BDNF) and are capable of internalizing bound factor. Neurotrophic factor binding induces autophosphorylation of an intracellular *trk* tyrosine kinase residue.

The Low-Affinity p75 Receptor

Chao and co-workers (1986) isolated the gene encoding human p75 using gene transfer assays with subsequent immunological detection of the transfected NGF receptor-expressing cells. The rat p75 gene was cloned by Radeke and co-workers (1987) using cDNA probes obtained from NGF-expressing cells by subtraction with excess mRNA from untransfected cells. Chicken p75 has been cloned by Large and co-

workers (1989). p75 mRNA size in chicken is 4.5 kb; in human, 3.8 kb; and in rat, 3.6 kb. The molecules differ at their 3' noncoding regions which accounts for the size differences.

The human p75 gene encodes a glycoprotein consisting of a single transmembrane polypeptide of 427 amino acid residues, 399 of which make up the mature protein. Rat p75 cDNA codes for a protein of 425 amino acids, including a 29 amino acid signal peptide. The extracellular domain comprises 222 amino acids, the transmembrane domain 22 amino acids, and the intracellular domain 152 amino acids. The amino acid sequences for human and rat p75 are almost identical but the chicken sequence has more variation. In all three species, the extracellular domain bears four conserved cysteine repeats. It has been demonstrated that these are responsible for ligand recognition since a secreted 168 amino acid receptor without all residues carboxy terminal to this domain still binds NGF (Welcher *et al.*, 1991; Yan *et al.*, 1991). There is also considerable homology between the three species within the transmembrane domain and in the C-terminus of the intracellular domain. The role of the intracellular domain remains to be fully elucidated. However, it has been shown that deletions in this region interfere with the ability of p75 to form functional high affinity NGF receptors (Hempstead *et al.*, 1989).

A soluble, truncated form of p75 has been isolated from conditioned media of p75-producing cells and in various biological fluids (Distefano and Johnson, 1988). This comprises the extracellular domain only and is thought to be produced by post-translational processing as opposed to differential splicing. The biological significance of the truncated receptor remains unclear, but it binds NGF, BDNF, and NT-3 with equal affinity to the full-length transmembrane receptor. Truncated p75 may limit the activity of neurotrophic factors by competing for factors with the full-length receptor or act as a neurotrophic factor transporter (review by Barker and Murphy, 1992).

trk Tyrosine Kinase Receptors

The human *trk* proto-oncogene locus was first identified when it became

malignantly active in a colon carcinoma patient (review by Barbacid *et al.*, 1991). Martin-Zanca *et al.* (1989) showed that this proto-oncogene encodes a 140 kD tyrosine kinase (designated $p140^{trkA}$) which exhibits the structural characteristics of a growth factor receptor (review by Chao, 1992). *In situ* hybridization studies using mouse embryos showed that the *trk*A gene is highly expressed in NGF-dependent neurons, for example, sensory neurons of spinal ganglia and a proportion of neurons from neural crest-derived cranial sensory ganglia (Martin-Zanca *et al.*, 1990). Further studies established that $p140^{trkA}$ is a component of the high affinity NGF receptor. NGF stimulates tyrosine autophosphorylation of $p140^{trkA}$ in PC12 cells, in spinal ganglia sensory neurons, and in NIH 3T3 cells transfected with *trk*A cDNA (Kaplan *et al.*, 1991a,b).

The observation that genes encoding *trk* tyrosine kinase receptors for BDNF and NT-3 should be structurally similar to that for $p140^{trkA}$ led to the isolation of cDNA clones for *trk*B and *trk*C by low stringency hybridization with *trk*A probes. The intracellular tyrosine kinase domains of *trk*A, *trk*B, and *trk*C share 85% sequence homology. *trk*B and *trk*C encode the tyrosine kinase receptors $p145^{trkB}$ and $p145^{trkC}$ respectively, both of molecular weight 145 kD. $p145^{trkB}$ has been shown to be the signalling receptor for BDNF (Klein *et al.*, 1991a; Glass *et al.*, 1991; Soppet *et al.*, 1991; Squinto *et al.*, 1991) and NT-4 (Berkemeier *et al.*, 1991; Ip *et al.*, 1992; Klein *et al.*, 1992). $p145^{trkC}$ has been shown to be the receptor for NT-3 (Lamballe *et al.*, 1991). There appears to be some cross-talk between NT-3 and $p140^{trkA}$ and $p145^{trkB}$. NT-3 induces *in vitro* tyrosine phosphorylation of these receptors with similar efficiency to their cognate ligands NGF and BDNF (or NT-4) respectively (Klein *et al.*, 1991b; Soppet *et al.*, 1991; Squinto *et al.*, 1991). Glass *et al.* (1991) have demonstrated that NT-3 induces tyrosine phoshorylation of these receptors much less efficiently in NIH 3T3 cells. Ip *et al.* (1993b) have shown that NT-3 exerts its neurotrophic effects on PC12 cells and cultured neuronal cells bearing $p145^{trkC}$ receptors.

Two isoforms of the *trk*A gene have been cloned. One was first identified in the

rat (Meakin *et al.*, 1992) and the other in the human (Martin-Zanca *et al.*, 1989). The isoforms differ in that the rat molecule codes for an additional 6 amino acids in the extracellular domain. They are produced by alternative splicing and both appear to generate functional receptors. However, the one coding for the additional 6 amino acids is expressed predominantly in neuronal tissues while the other is expressed mainly in non-neuronal tissues (Barker *et al.*, 1993). The p140^{trkA} receptor comprises a glycosylated peptide chain with a single transmembrane domain (review by Meakin and Shooter, 1992). It is larger than p75, containing approximately 790 amino acid residues.

Middlemas and co-workers (1991) have shown that the rat *trk*B gene expresses multiple transcripts. Two of these are predicted to encode the full-length receptor and are 9.0 and 4.8 kb in size. There are a number of C-terminally truncated transcripts of 7.5, 7.0, 2.4, 0.9, and 0.7 kb in length. These possess the extracellular domain but lack the tyrosine kinase domain. A 1.6 kb mRNA was also isolated that comprises the tyrosine kinase domain only. These forms are possibly generated through alternative splicing. Northern blot analysis has shown that at least 5 *trk*B transcripts are present in the chick (Déchant *et al.*, 1993a). These are estimated to be 9.0, 8.1, 6.3, 5.0, and 1.6 kb in length. The 9.0 kb transcript is the only one possessing a tyrosine kinase domain and has only been detected in neuronal structures. The shorter transcripts lacking the tyrosine kinase domain are expressed predominantly in non-neuronal tissues.

Lamballe and co-workers (1991) first described cDNA clones encoding porcine p145^{trkC}. More recently, Valenzuela and co-workers (1993) have identified a number of rat *trk*C cDNA clones encoding novel forms of p145^{trkC}. These include clones encoding both a full-length receptor similar to that identified in pig and 4 truncated forms lacking the tyrosine kinase domain. In addition, cDNAs encoding forms of p145^{trkC} with either 14 or 39 amino acid inserts in their tyrosine kinase domains have been identified. All forms appear to have been generated by alternative splicing. The major transcript encoding the full-length receptor is 14 kb in size (as shown by Northern blotting). Although all forms are expressed throughout the nervous system,

only the truncated ones are detected in astrocytes, peripheral nerves, and non-neuronal tissues. Those containing the amino acid inserts are still capable of autophosphorylation in response to NT-3 but cannot mediate proliferation in fibroblasts or neuronal differentiation in PC12 cells.

Developmental Expression of *trk* Receptors

*trk*A

In mouse, *trk*A is initially expressed during early neurogenesis at embryonic day (E) 9.5 (Martin-Zanca *et al.*, 1990; Wyatt and Davies, 1993). By E13.5, its expression is confined to certain structures of the nervous system. These include the DRG and cranial sensory ganglia (for example, the trigeminal, superior, and jugular ganglia). In older embryos (E17.5), *trk*A expression is also detectable in sympathetic ganglia (Martin-Zanca *et al.*, 1990) and in defined structures of the central nervous system, for example, the cholinergic neurons of the basal forebrain and the striatum (Steininger *et al.*, 1993).

*trk*B

Mouse *trk*B expression is first detected in both the central and peripheral nervous system at E8.5. It is expressed in many structures, for example, the forebrain, caudal midbrain, hindbrain, spinal cord, trigeminal ganglion, and differentiating neural crest cells which form the dorsal root ganglia (Klein *et al.*, 1990b). *trk*B expression is found to be widespread in the nervous system throughout embryonic development.

In adult mouse, the highest expression levels are found in the brain and spinal cord (Klein *et al.*, 1990b). Expression is also detected in most structures of the cerebrum including the cortical layers, thalamus, and hippocampus. Non-neuronal tissues express *trk*B, for example, tongue, whisker pad mesenchyme, and outer dermal papilla (Klein *et al.*, 1990a).

There are highly specific patterns of expression for the full-length catalytic receptor and the truncated forms without a tyrosine kinase domain (Klein *et al.*, 1990a).

In situ hybridization studies have shown that the truncated transcripts are located predominantly in the choroid plexus and ventricles. In general, the full-length receptor is more abundant in non-neuronal cells, for example, astrocytes, oligodendrocytes, and Schwann cells (Frisen *et al.*, 1993).

In chicken, *trk*B transcripts encoding the full-length receptor have been detected during early embryonic development in most sensory ganglia. No expression is detected at any stage in BDNF non-responsive sympathetic and ciliary neurons (Déchant *et al.*, 1993a). Expression is first detected in the trigeminal ganglion at E3.5. At E5, expression is restricted to the ventral region of the ganglion; expression in the dorsal region is poor at early stages of development but increases at later stages. In the nodose ganglion, transcripts are first detected at E4. Expression in the vestibular ganglion is high throughout development. In the DRG, expression is first detected at the mid-thoracic level at E3.5, predominantly in the ventral and lateral regions of the ganglion. Expression is also detected at lower levels in the ventral horn of the spinal cord, particularly in areas with motoneurons. Transcripts encoding truncated receptors have been found in non-neuronal structures, for example, mesenchymal cells surounding the spinal cord.

*trk*C

The *trk*C locus encodes at least eight transcripts. To date, most expression studies have been carried out using probes that recognize all forms. In mouse, transcripts are first detected at E7.5 in neuroectoderm, where the expression is low (Tessarollo *et al.*, 1993). By E9.5, they are also detectable in developing DRG, the telencephalon, and the spinal cord (Tessarollo *et al.*, 1993; Lamballe *et al.*, 1994). At E11, expression is widespread throughout the central nervous system and in certain structures in the peripheral nervous system, for example, the DRG. At E13.5, it is now more widespread in the peripheral nervous system, noticeably in the trigeminal and otic ganglia (Lamballe *et al.*, 1994) and remains extensive at E15.5 in both the central and peripheral nervous systems. In the latter, expression is evident in the vestibular-

acoustic, nodose, and geniculate cranial sensory ganglia. *trk*C transcripts have also been located in structures outside the nervous system, for example, facial structures including vibrissae of the snout, dental papillae, and posterior tongue, and body cavity structures such as the submandibular gland and the walls of arteries and urinogenital tracts. In adult mouse, *trk*C transcripts can be detected in many structures of the central nervous system (Lamballe *et al.*, 1994).

The Role of p75 in Neurotrophic Factor Binding and Responsiveness

The role of p75 in mediating the effects of neurotrophic factors remains unclear and the available evidence is conflicting. Weskamp and Reichardt (1991) have shown that a mutated NGF with an impaired p75 binding site is still capable of inducing neurite outgrowth and cell survival in PC12 cells and primary cultures of neurons. Also, blocking the ligand binding domain of p75 using antibodies has shown that p75 is not necessary for NGF action. However, other studies in which p75 has a deletion mutation have shown that high-affinity binding is abolished unless intact p75 is co-expressed with p140^{trkA} (Hempstead *et al.*, 1990; Battleman *et al.*, 1993).

There is an indication that the number of high-affinity bnding sites formed is influenced not simply by the co-expression of p75 and p140^{trkA} but by whether these molecules are co-expressed in the relevant ratio. p75 expression in NGF-responsive neurons is markedly higher than that of *trk*A. For example, in PC12 cells and neonatal sympathetic ganglia, p75 mRNA levels are five- to ten-fold in excess of *trk*A mRNA levels (review by Chao, 1994). Also, Hempstead and co-workers (1992) have shown that overexpression of *trk*A in PC12 cells increases the number of high- and low-affinity binding sites, indicating that neurotrophic factor binding is influenced by the levels of both p75 and *trk* components.

The nature of the p75-*trk* complex is uncertain. Hempstead and co-workers (1991) proposed a heterodimeric model in which the cytoplasmic domain of p75 interacts with the *trk* protein tyrosine kinase, giving rise to a high-affinity NGF binding site. Jing and co-workers (1992) have established that homodimerization of *trk*A

receptors is necessary for NGF binding to activate them. Given that a certain ratio of p75 to *trk* may be required to determine neurotrophic factor specificity, the numbers of p75 and *trk* molecules involved in the functional receptor are likely to be complex. A model in which p75 first binds neurotrophic factors and then presents and transfers them to adjacent *trk* receptors has not been substantiated.

As p75 binds all neurotrophic factors, it has been suggested that it might be involved in the process by which the *trk* receptors show specificity for certain factors (Rodriguez-Tébar *et al.*, 1992). For example, p75-deficient fibroblasts transfected with *trk*B will respond to BDNF, NT-3, and NT-4/5, whereas p75-expressing PC12 cells transfected with *trk*B will only respond to BDNF (Ip *et al.*, 1993b). These results indicate that the *trk* receptors may respond to different neurotrophic factors more selectively when p75 is also expressed. The co-expression of p75 with $p140^{trkA}$ can also result in increased sensitivity to neurotrophic factors (Scheibe and Wagner, 1992; Davies *et al*., 1993b).

Using mice with a null mutation for p75, Lee and co-workers (1992) gained further insight into the role of p75 during embryonic development. These animals showed extensive loss of cutaneous sensory neurons along with a lack of sympathetic innervation in certain targets, especially the pineal gland and a subset of footpad sweat glands (Lee *et al.*, 1994a). The effect of the p75 null mutation on sensory neuron survival has been studied in primary cultures of trigeminal neurons from mice homozygous for the mutation (Davies *et al.*, 1993b). These neurons were less sensitive to NGF compared with wild-type neurons, although the survival responses to BDNF or NT-3 were not affected. This suggests that both p75 and *trk*A play an important role in regulating the sensitivity of these neurons to NGF during development. Neuronal survival may be controlled through receptor expression, with the p75 to *trk* ratio providing a means of modulating receptor specificity to neurotrophic factors.

Signal Transduction by the *trk* Receptor

Signal transduction is the transmission of growth factor signalling from the

receptor to the nucleus. To date, much of the work with neurotrophic factors has concentrated on NGF signalling, using mostly PC12 cells. The binding of NGF to the *trk*A receptor initiates the tyrosine kinase activity of the receptor, leading to tyrosine phosphorylation of the receptor and intracellular proteins. This provides a means of regulating cellular protein activity and initiates gene transcription, which in turn controls the growth, migration, morphology, and survival of neurons.

The binding of NGF to *trk*A is believed to induce the formation of *trk*A dimers or oligomers with subsequent transphosphorylation on receptor tyrosine residues (Jing *et al.*, 1992). As discussed previously, it appears that p75 may play a role in regulating neuronal specificity and sensitivity to neurotrophic factors. Receptor transphosphorylation plays a dual role. It firstly activates the receptor and, secondly, provides recognition sites for the binding of cellular signalling proteins. NGF induces phosphorylation on human *trk*A on at least five tyrosine residues. Two of these phosphotyrosines bind at least two intracellular proteins involved in the NGF signalling process. These include the SHC protein group and phospholipase C-γ1 (PLC-γ1) (Stephens *et al.*, 1994). These proteins contain in their non-catalytic regions an approximately 100 amino acid domain referred to as src homology 2 or SH2 (hence the abbreviation SHC for the former) which enables them to complex with specific sequences of receptors with phosphorylated tyrosine residues (Songyang *et al.*, 1993). The SHC gene encodes three proteins of similar size and sequence and these are the major substrates for NGF-induced tyrosine kinase activity in PC12 cells. Other proteins complexing with the *trk*A receptor include phosphatidylinositol-3 kinase (PI-3 kinase) and extracellular signal-regulated kinase 1 (Erk 1) (Loeb *et al.*, 1992).

The receptor-protein complex is an important intermediate in growth regulatory signalling. The purpose of complex formation may be two-fold. It may provide a means of recruiting proteins from the cytoplasm to the plasma membrane (Cantley *et al.*, 1991). For example, the phosphatidylinositol phospholipid substrates of PI-3 kinase are found in the plasma membrane and complex formation may bring enzyme and substrate in closer proximity. The complexing of signalling protein and receptor

may also serve to activate the protein by inducing phosphorylation on tyrosine residues, as has been found for PI-3 kinase, PLC-γ1, and the SHC proteins (Shoelsen *et al.*, 1991).

The Roles of PLC-γ1, PI-3 Kinase, SHC Proteins, and Erk 1

The activities of PLC-γ1, PI-3 kinase and the SHC proteins are stimulated in response to NGF binding to the *trk*A receptor. PLC-γ1 catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacylglycerol (DG) and inositol trisphosphate (IP_3), both of which are important second messengers (Rhee and Choi, 1992). DG activates protein kinase C and IP_3 causes a transient increase in intracellular calcium levels. Other functions associated with PLC-γ1 include changes in ion fluxes and intracellular pH, cytoskeletal rearrangements, and stimulation of gene transcription (review by Kaplan and Stephens, 1994).

In PC12 cells and fibroblasts, PI-3 kinase catalyzes the formation of phosphoinositides (Whitman *et al.*, 1987). The precise role of these second messengers remains uncertain. In mammalian cells, they may mediate the transport of activated tyrosine kinase receptors to specific intracellular sites (Kapellar *et al.*, 1993).

SHC proteins induce neurite outgrowth when overexpressed in PC12 cells (Rozakis-Adcock *et al.*, 1992). They also play a part in activating the Ras protein (a later target of *trk*A activity to be discussed in the following section) (Suen *et al.*, 1993).

Erk 1 is a serine/threonine kinase and part of the Ras signal transduction pathway. The *trk*A receptor associates with activated forms of Erk 1 which are not phosphorylated on tyrosine residues and so is not one of its direct substrates.

The Ras Signal Transduction Pathway

Signalling events occurring downstream of PLC-γ1, the SHC proteins, and PI-3 kinase involve a small p21Ras G protein, activators of Ras, and serine/threonine kinases activated either directly or indirectly by Ras activity (Li *et al.*, 1992). Stimulated *trk*A receptors bind to and induce tyrosine phosphorylation and activation of PLC-γ1,

PI-3 kinase, and the SHC proteins. The SHC proteins then bind to Grb2 (another protein bearing src homology regions) and SOS (son of sevenless). The latter is a protein serving as an exchange factor for the activation of p21Ras. It enhances the exchange of GDP against GTP on the p21Ras protein which oscillates between inactive GDP-bound and active (signalling) GTP-bound forms (McCormick, 1994). The activation of p21Ras by exchange factors or by inhibition of RasGTP-ase activating proteins (GAP) is also involved in raising levels of GTP-bound p21Ras during NGF activity in PC12 cells (Li *et al.*, 1992).

In PC12 cells, the signal transduction cascade from the cytoplasm (with activated p21Ras) to the nucleus is a sequence of phosphorylation events which leads to fibre outgrowth. This involves the serine/threonine kinase proto-oncogene *Raf* (c-*Raf*, b-*Raf*) which activates mitogen-activated protein kinase kinase (MAPKK) by serine phosphorylation. MAPKK then activates mitogen-activated protein kinase (MAPK) by phosphorylation on threonine and tyrosine residues. In PC12 cells, MAPK comprises the extracellular signal-related kinases Erk 1 and Erk 2 (review by Heumann, 1994).

There is evidence for a signal transduction pathway that functions independently of Ras, involving SNT (*suc*-associated neurotrophic factor-induced tyrosine phosphorylated target). This is a nuclear protein that appears to be tyrosine phosphorylated in NGF-treated PC12 cells and in neurotrophic factor-treated neuronal cultures (Rabin *et al.*, 1993). Studies using PC12 cells expressing *trk*A mutants which prevent Ras signalling but not SNT tyrosine phosphorylation, suggest that SNT forms part of a signalling cascade that by-passes Ras (Stephens *et al.*, 1994).

Neurotrophic Factors and Nervous System Development

Until recently, the principal means of studying neurotrophic factors has been by employing blocking antibodies to neutralize their functioning. Although the information thus provided has proved valuable, the structural similarities between the factors has brought into question the specificity of such antibodies. Also, studies of the central nervous system have been ineffective since antibodies penetrate the blood-brain barrier

poorly. More recently, gene targeting has confirmed the role of neurotrophic factors in nervous system development through the production of mice that have a null mutation in genes encoding either the factors or their receptors (the so-called 'knockout' mice).

It is now clear that neurotrophic factors have a complex role in shaping the developing nervous system. They not only promote the survival of developing neurons but regulate the proliferation and differentiation of neuron progenitor cells and determine other neuronal characteristics throughout life. A neuronal population may respond to more than one neurotrophic factor or may depend on different factors at different stages of development.

Proliferation and Differentiation of Neuronal Progenitors

NGF has been shown to influence *in vitro* proliferation of progenitor cells from embryonic rat striatum (Cattaneo and McKay, 1990). However, studies of striatal neurons from NGF 'knockout' mice (Crowley *et al.*, 1994) and *trk*A 'knockout' mice (Smeyne *et al.*, 1994) have indicated that NGF may not be involved in regulating the proliferation of neuronal precursors since the resultant neuronal population in the null mutant animals is not diminished in compared to those in wild type animals. Further studies of different neuronal populations from these mice should confirm any possible proliferative roles for NGF.

Wright *et al.* (1992) have shown that NT-3, and not NGF or BDNF, increases the number of differentiating neurons in cultures of early DRG cells. This suggests that NT-3 may induce proliferation and/or differentiation of sensory neuron progenitor cells from the neural crest. These progenitor trunk neural crest cells express *trk*C mRNA *in vivo* during migration (Tessarollo *et al.*, 1993) and undergo *in vitro* proliferation in response to NT-3 (Kalcheim *et al.*, 1992). However, it has not been ascertained whether NT-3 influences the proliferation of multipotent neural crest cells or neural crest cells that are committed to a particular differentiation pathway. Gaese *et al.* (1994) have studied the effect of using anti-NT-3 to neutralize the function of endogenous NT-3 in the placode-derived nodose ganglion and in DRG during gangliogenesis. They

found a marked decrease in the number of neurons in these ganglia. This suggests that NT-3 may play a role in maintaining these neurons prior to their receiving target-derived influences, or it may be that NT-3 regulates neuronal proliferation during gangliogenesis.

Kalcheim and Gendreau (1988) have studied possible roles for NGF and BDNF in promoting differentiation of neuronal precursors. Using the expression of substance P as a marker for sensory neurons, they found that the number of these neurons increased in response to BDNF and not NGF in cultures of post-migratory trunk neural crest cells. This indicates that BDNF may play a part in the differentiation and/or survival of neural crest-derived sensory neurons. Using expression of the SSEA 1 epitope as a marker for sensory neurons, studies by Sieber-Blum (1991) in the quail have suggested that BDNF directs pluripotent neural crest cells to differentiate along the primary sensory neuron lineage. However, it required three weeks of exposure to BDNF for the proportion of cells expressing SSEA 1 to increase significantly, while, *in vivo,* neuronal differentiation takes place a few days after neural crest migration. It is possible, therefore, that these results may not be developmentally relevant. In mice with a null mutation in the BDNF gene (Ernfors *et al.*, 1994a; Jones *et al.*, 1994) or the *trk*B gene (Klein *et al.*, 1993), there are marked reductions in the sensory neurons, though these may be attributed to losses due to poor survival. To determine whether differentiation plays a part in reducing neuronal numbers in these mice, further detailed studies must be carried out to find out when the reduction takes place.

Early Neuronal Maturation

Wright *et al.* (1992) have observed that early DRG neurons undergo a maturational change in culture; at first they have small, spindle-shaped, phase-dark cell bodies which become spherical and phase-bright, extending long neurites. This change takes place in chemically defined medium within 24 hours of the cells being placed in culture. Very low concentrations of BDNF and NT-3 were found to accelerate this maturational process, while NGF had no effect. *In vivo*, these neurons have not

innervated their targets and are not in contact with target-derived neurotrophic factors, being independent of neurotrophic factors for survival. These early DRG neurons do, however, express BDNF mRNA which indicates that they may promote maturation by producing their own BDNF (Wright *et al.*, 1992). BDNF may be acting via an autocrine mechanism since early DRG neurons still mature when cultured in isolation and maturation is inhibited by antisense BDNF oligonucleotides (Wright *et al.*, 1992).

The Onset of Neurotrophic Factor Responsiveness

Prior to innervating their targets, neurons may survive and extend axons without neurotrophic factor support. Evidence for this has come from studies using sensory ganglion explants (Davies *et al.*, 1981; Davies and Lumsden, 1984). Neurites emerge from these explants in neurotrophic factor-free medium at the stage when the ganglia extend axons towards their targets *in vivo*. In dissociated cultures of early sympathetic (Ernsberger *et al.*, 1989a) and sensory ganglia (Ernsberger and Rohrer, 1988), in which few non-neuronal cells are present, neurons survive independently of neurotrophic factors, ruling out the possibility that early neurons in ganglion explants might obtain neurotrophic support directly from non-neuronal cells. Wright *et al.* (1992) have shown that early sensory neurons survive without neurotrophic factors in single cell cultures in defined medium and, therefore, do not obtain neurotrophic support from other cells or from factors in poorly defined serum supplements.

There is also a possibility that these early neurons are dependent on neurotrophic factors for survival but receive them via an autocrine mechanism. A number of studies have shown that some developing neurons do express BDNF mRNA and/or NT-3 mRNA (Ernfors *et al.*, 1992; Schecterson and Bothwell, 1992). To date, however, the only study showing firm evidence of an autocrine mechanism in developing neurons is of one promoting neuronal maturation in early DRG neurons and not survival (Wright *et al.*, 1992). Although antisense BDNF oligonucleotides delay a maturational change in these neurons, they do not have any effect on survival.

The period of neurotrophic factor dependency is believed to commence when

neuronal axons reach their targets. Evidence for this comes from studies by Davies and Lumsden (1984) in which trigeminal ganglion explants start showing extensive neurite outgrowth in response to NGF at about the stage when trigeminal neurons begin innervating their peripheral targets *in vivo*. More recent studies by Buchman and Davies (1993) have shown that early trigeminal neurons in low-density cultures survive independently of neurotrophic factors at the stage when their axons are growing towards their targets *in vivo* and become neurotrophic factor dependent at the stage when their peripheral axons reach their targets *in vivo*. NGF dependency is preceded by a short period of dependency on BDNF, NT-3, or NT-4/5 (Buchman and Davies, 1993; Davies *et al.*, 1993a).

That neurons only become neurotrophic factor dependent when their axons reach their targets has been further substantiated by studying other types of cranial sensory neurons. Extensive *in vitro* work has been carried out using embryonic chicken vestibibular, geniculate, petrosal, and nodose ganglia. These ganglia are derived from thickened regions of head ectoderm (the neurogenic placodes) and are born during the same period in development. Their axons, however, have different distances to grow before reaching their central and peripheral targets and this is reflected in differences in their ability to survive in culture without BDNF before becoming BDNF dependent. Vestibular neurons have the closest targets and only survive for a short time without BDNF. Nodose neurons have the most distant targets and can survive the longest time in the absence of BDNF. Geniculate and petrosal neurons have intermediate target distances and survive without BDNF for intermediate times (Vogel and Davies, 1991).

Evidence for the Intrinsic Timing of Responsiveness in Neurons

If the cue for the onset of neurotrophic factor responsiveness were entirely dependent upon axons reaching their targets, then those axons which fail to do so would continue growing and not be eliminated by cell death. The above mentioned work of Vogel and Davies (1991) using low-density cultures of cranial sensory neurons has provided evidence that neurons may possess an intrinsic programme for

timing the onset of neurotrophic factor dependency. This would presumably ensure that neurons do not survive if their axons fail to reach their targets. The onset of BDNF responsiveness in early nodose neurons can be brought forward in time by transiently exposing them to BDNF within 24 hours of when they would be expected to start responding to BDNF *in vitro* (Vogel and Davies, 1991). It seems likely that neurons innervate their targets within a 'window' of neurotrophic factor independence and become responsive partly under the influence of target-derived factor- the duration of this 'window' being controlled by an intrinsic timing programme.

Neurotrophic Factor Requirements During Early Stages of Target Innervation

The neurotrophic factor survival requirements of some neurons during the early stages of target field innervation has proved more complex than first thought. These have been studied extensively using cultured embryonic mouse trigeminal neurons (Buchman and Davies, 1993; Davies *et al.*, 1993a). It appears that these neurons respond to different neurotrophic factors at different stages during this period of development. When placed in low density culture in defined medium at the time when their axons would normally reach their peripheral targets *in vivo*, they require BDNF, NT-3, or NT-4/5 to survive but not NGF. After a few days of further development, they lose their responsiveness to BDNF, NT-3, and NT-4/5 and become responsive to NGF. Buchman and Davies (1993) have clearly demonstrated that the same neurons initially responsive to BDNF, NT-3, and NT-4/5 become NGF responsive. This observation has been further substantiated by examining the trigeminal ganglia of neonate mice with a null mutation in the*trk*B gene. The considerable neuronal loss cannot be accounted for simply by the elimination of those neurons requiring BDNF for survival during the period of naturally occurring neuronal death as only a small proportion are ultimately BDNF-responsive (Klein *et al.*, 1993). It is possible that the increased cell loss results from death occurring during the earlier stage of BDNF or NT-4/5 responsiveness.

There is *in vitro* evidence that cells of the sympathetic lineage also change neurotrophic factor survival requirements early in development. Many proliferating sympathetic neuroblasts survive in the absence of neurotrophic factors (DiCocco-Bloom and Black, 1988). However, NT-3 and not BDNF or NGF has been shown to increase the survival of these cells (DiCocco-Bloom *et al.*, 1993). Later in development, sympathetic neurons require NGF for survival, while only much higher concentrations of NT-3 are effective (Déchant *et al.*, 1993b). It has not been directly demonstrated whether sympathetic neuroblasts/neurons undergo a change in survival requirement from NT-3 early in development to NGF in later development. However, the fact that neonatal mice with a null mutation in the NT-3 gene possess only half the number of neurons in their sympathetic ganglia compared with wild type mice provides further evidence for this (Ernfors *et al.*, 1994b). NGF-responsive jugular neurons are also transiently supported by BDNF and NT-3 early in their development (Buj-Bello *et al.*, 1994). *In vitro,* embryonic DRG neurons are initially neurotrophic factor independent but subsequently respond to either NGF or BDNF before a proportion of the neurons lose their BDNF-responsiveness (Ernsberger and Rohrer, 1988). It is not known whether early DRG neurons are transiently BDNF responsive before becoming NGF responsive, but *in vivo* studies have shown that BDNF and not NGF supports early DRG neurons (Kalcheim *et al.*, 1987).

However, BDNF-responsive embryonic chicken ventrolateral trigeminal neurons do not respond to NGF or NT-3 in early development. Similarly, BDNF- and NT-3-responsive nodose neurons do not show an early response to NGF (Buj-Bello *et al.*, 1994). However, it is possible that these neurons are supported by other survival factors early in development.

The reason why some neurons respond to different neurotrophic factors at different stages of development is not known for certain. For mouse trigeminal neurons *in vivo*, it appears that the supply of BDNF, NT-3, and NT-4/5 is not limiting for early survival so the purpose of the period of responsiveness to these factors could be to maintain survival prior to the NGF-responsive stage, during which neuronal numbers

are regulated by naturally occurring cell death. The initial period of responsiveness to BDNF, NT-3, and NT-4/5 may be to delay neuronal death until most of the axons have reached their targets so that a maximum number of neurons can compete for NGF simultaneously, giving a greater selection.

Neurotrophic Factor Requirements During Neuronal Death

The availability of neurotrophic factors to developing neurons during naturally occurring cell death determines the final size of a neuronal population. Neurotrophic factor requirements of neurons during this period have been studied in culture and *in vivo* by the use of blocking antibodies to factors and gene targeting to 'knockout' either the factors or their receptors. These give further insight into the role of neurotrophic factors in controlling neuronal survival.

NGF Responsive Neurons

For sympathetic ganglia, the period of neuronal death occurs a few days after birth as their time course of development is delayed compared to that for sensory neurons, which undergo cell death before birth. The first demonstration that sympathetic neurons require NGF for survival came from the work of Levi-Montalcini (1987) in which anti-NGF antiserum was injected into neonatal rodents, destroying the paravertebral sympathetic chain. This observation has been confirmed by studies using mice with null mutations in the NGF (Crowley *et al.*, 1994) and *trk*A genes (Smeyne *et al.*, 1994). In these mice, the sympathetic chain has been almost completely eliminated by 10 to 14 days after birth.

A subset of DRG neurons and some cranial sensory ganglion neurons (e.g., trigeminal neurons in the mouse, dorsomedial trigeminal neurons in the chicken, and jugular neurons) require NGF for survival . These are believed to be the small-diameter neurons thought to mediate pain (nociceptive) and thermal receptive functions. NGF requirement of DRG neurons has been studied by exposing foetuses to anti-NGF *in utero* (Johnson *et al.*, 1990; Ruit *et al.*, 1992). After birth, the pups were unresponsive

to painful stimuli and showed selective loss of small-diameter DRG neurons. The study by Davies and Lindsay (1985) has shown that only small-diameter cutaneous cranial sensory neurons respond to NGF *in vitro*.

The observation that NGF is necessary for the survival of nociceptive and thermal receptive neurons is borne out by the phenotype of mice with null mutations in the NGF and *trk*A genes (Crowley *et al.*, 1994; Smeyne *et al.*, 1994). After birth, these mice are insensitive to painful and thermal stimuli and show selective loss in small-diameter DRG neurons. Conversely, transgenic mice overexpressing NGF exhibit extreme hyperalgesia to noxious mechanical stimulation (Davis *et al.*, 1993). Mice with a null mutation in the p75 gene have an impaired ability to detect thermal stimuli, indicating a loss in nociceptive and thermal receptive neurons (Lee *et al.*, 1992). This strengthens the idea that p75 acts to modulate the functions of *trk*A, possibly increasing neuronal responsiveness to NGF during critical periods in development.

There are several studies which suggest that basal forebrain cholinergic neurons require NGF for survival. It has been shown that NGF infusion rescues these neurons from death after axotomy (Hefti, 1986; Montero and Hefti, 1988) and that NGF promotes their survival in low density culture (Hartikka and Hefti, 1988a,b). However, these observations do not agree with the findings of studies using mice with null mutations in the NGF and *trk*A genes (Crowley *et al.*, 1994; Smeyne *et al.*, 1994). These mice appear to have suffered no reduction in the number of these neurons. Instead, basal forebrain cholinergic neurons from mice with a null mutation in the NGF gene stain more lightly for the transmitter enzyme choline acetyltransferase (ChAT) than those of wild type mice (Crowley *et al.*, 1994). This is in agreement with previous studies showing that NGF increases (Hartikka and Hefti, 1988a) and anti-NGF decreases (Vantini *et al.*, 1989) ChAT expression in these neurons. Mice with a null mutation in the *trk*A gene show a considerable reduction in axonal acetylcholinesterase activity in the hippocampal and cortical target fields of the basal forebrain cholinergic neurons (Smeyne *et al.*, 1994). It appears, therefore, that these neurons need NGF not for survival but for regulating the synthesis of transmitter enzymes.

BDNF Responsive Neurons

BDNF supports the survival of proprioceptive neurons from the trigeminal mesencephalic nucleus (TMN) and proprioceptive neurons in DRG, which relay information about joint movement and position (Davies *et al*., 1986a,b; review by Davies, 1992). Neurons from the vestibular, geniculate, petrosal ganglia, from the ventrolateral region of the trigeminal ganglion in the chicken, and a sub-population of nodose ganglion neurons are also BDNF-responsive (review by Davies, 1992). BDNF has been shown to promote the survival of motoneurons innervating skeletal muscle. BDNF applied to chicken embryos *in ovo* at the time of naturally occurring death rescues approximately one third of the motoneurons that normally die during this phase (Oppenheim, 1992). This is not observed with NGF. Axotomized motoneurons in neonatal rats can be partially rescued from death by the local application of BDNF, but not NGF, to the cut nerve (Koliatsos *et al.*, 1993). *In vitro* studies have indicated that several populations of neurons in the central nervous system are responsive to BDNF, including dopaminergic neurons in the midbrain and granule cells in the cerebellum (review by Korsching, 1993). The high levels of BDNF expression in hippocampal and cortical neurons suggest that BDNF might play a role in their development (Ernfors *et al.*, 1990a; Phillips *et al.*. 1990).

Studies using mice with null mutations in the BDNF (Jones *et al.*, 1994) and *trk*B (Klein *et al.*, 1993) genes have provided more detailed information about the role of BDNF in the development of these neurons. *trk*B 'knockout' mice die soon after birth. Presumably, because *trk*B is the receptor for both BDNF and NT-4/5, the mutation is more deleterious to nervous system development. BDNF 'knockout' mice exhibit behaviour consistent of an abnormally functioning inner ear related to balance. The vestibular ganglia innervating this region is very depleted of neurons, confirming the importance of BDNF in their survival. In both BDNF and *trk*B 'knockout' mice, there are significant though not massive neuronal losses in the trigeminal ganglia, consistent with these neurons being transiently responsive to BDNF before becoming NGF dependent (Buchman and Davies, 1993). There is significant depletion in

proprioceptive TMN neurons in BDNF 'knockout' mice and also some loss of DRG neurons with both 'knockouts', in agreement with the observation that BDNF supports a proprioceptive sub-population of these (review by Davies, 1992). This deficit in proprioceptive neurons might contribute to some of the movement abnormalities observed in BDNF 'knockout' mice. There is a significant reduction in the number of nodose ganglion neurons. The other BDNF-responsive cranial sensory neurons have not been studied in these mice.

Surprisingly, motoneurons in BDNF 'knockout' mice are not affected (Jones *et al.*, 1994), although previously mentioned studies have shown that this factor promotes their survival (Oppenheim, 1992). *trk*B 'knockout' mice, however, exhibit a significant loss in motoneurons (Klein *et al.*, 1993). This is consistent with the study of Henderson *el al.* (1993) which shows that NT-4/5 promotes motoneuron survival. This suggests that NT-4/5 may be more important for motoneuron survival *in vivo* than BDNF.

The neuronal populations in the central nervous system thought to be BDNF responsive (midbrain dopaminergic neurons, cerebellar granule cells, and hippocampal and cortical neurons) are present in normal numbers in both BDNF and *trk*B 'knockout' mice (Jones *et al.*, 1994; Klein *et al.*, 1993). All cortical layers are developed but thinner than in wild type animals and the overall morphology of the hippocampus appears normal. There is, however, evidence to suggest that BDNF may play a role in regulating neuronal functioning in the central nervous system (Jones *et al.*, 1994). Cortical interneurons that use the neurotransmitter γ-aminobutyric acid (GABA) express neuropeptide Y (NPY) which is a member of the neuropeptide family of neuroendocrine hormones. In BDNF 'knockout' mice, NPY expression in these neurons is reduced while the number of neurons positive for GABA appears normal. This suggests that BDNF may regulate peptide expression in the central nervous system and not neuronal survival, a function it may have in common with NGF. However, BDNF 'knockout' mice only live for a few weeks so it cannot be ruled out that NPY expression might have increased at a later stage of postnatal central nervous system

development, or perhaps the role of BDNF is not one of regulating expression but of directing development along certain cell lineages (Snider, 1994). The levels of the calcium binding proteins parvalbumin and calbindin are also reduced in the cortical and hippocampal regions of BDNF 'knockout' mice and so BDNF may regulate these (Snider, 1994). Calcium binding proteins buffer increased intracellular calcium levels caused by neuronal activity (Baimbridge *et al.*, 1992).

NT-3 Responsive Neurons

In vitro studies have shown that NT-3, in addition to BDNF, promotes the survival of proprioceptive neurons in the TMN and a proprioceptive neuronal sub-population in DRG (Hohn *et al.*, 1990; Hory-Lee *et al.*, 1993). Studies using mice with null mutations in the NT-3 (Ernfors *et al.*, 1994b) and *trk*C (Klein *et al.*, 1994) genes have confirmed these *in vitro* observations. For both null mutations, the animals show a phenotype of abnormal postures and movements, indicative of deficits in proprioception. DiI labelling techiques have shown that the *trk*C 'knockout' mice have no DRG axon collaterals (group 1a afferents) projecting to the spinal cord (Klein *et al.*, 1994), while the NT-3 'knockout' animals have only a few (Ernfors *et al.*, 1994b). DRG in NT-3 'knockout' mice have reduced staining for carbonic anhydrase and parvalbumin, which are markers for proprioceptive DRG neurons (Ernfors *et al.*, 1994b). Also, muscle spindles (sensory end organs involved in proprioception) fail to develop in these animals (Ernfors *et al.*, 1994b). Animals heterozygous for the NT-3 mutation show a 50% reduction in the number of muscle spindles which further illustrates that neurotrophic factors are present in limiting quantities during development. NT-3 'knockout' mice also have a significant reduction in proprioceptive TMN neurons (Ernfors *et al.*, 1994b).

NT-3, like NGF, promotes the survival of post-mitotic foetal and neonatal sympathetic neurons *in vitro,* but higher concentrations are required compared to NGF (Davies *et al.*, 1993b; Déchant *et al.*, 1993b; Lee *et al.*, 1994b). The number of sympathetic neurons present in NT-3 'knockout' mice is approximately half that of wild

type animals (Ernfors *et al.*, 1994b). It has not been verified whether this reduction is due to the increased death of post-mitotic sympathetic neurons. Kalcheim *et al.* (1992) have shown that NT-3 is a mitogen for neural crest cells *in vitro* and so the loss in sympathetic neurons in NT-3 'knockout' mice could be due to the reduced development of sympathetic neuronal precursors. Also, a proportion of proliferating sympathetic neuronal precursors are supported by low concentrations of NT-3 *in vitro* (Birren *et al.*, 1993; Déchant *et al.*, 1993b; DiCicco-Bloom *et al.*, 1993) and so the loss of sympathetic neurons could be due to the poor survival of these precursors.

Henderson *et al.* (1993) have shown that NT-3 promotes the survival of embryonic rat motoneurons *in vitro*. NT-3 'knockout' mice have apparently normal numbers of facial nucleus motoneurons (Ernfors *et al.*, 1994b), while the spinal nerve roots of *trk*C 'knockout' mice show a significant loss of motoneurons (Klein *et al.*, 1994), as determined by myelinated axon counts. This may be indicative of the different neurotrophic factor requirements of cranial and spinal motoneurons. However, there is a possibility that any reduction in motoneuron number might be due to the lack of group 1a afferents in NT-3 and *trk*C 'knockout' mice as these fibres are thought to play a part in promoting motoneuron survivial (Okado and Oppenheim, 1984).

NT-4/5 Responsive Neurons

Davies *et al*. (1993a) have shown that human recombinant NT-4/5 is a survival factor for distinct populations of mammalian sensory neurons during both the early stages of target field innervation and the period of naturally occurring cell death. NT-4/5 transiently promotes the survival of mouse trigeminal and jugular neurons during the early stage of target field innervation prior to naturally occurring cell death. At this time, these neurons are known to be transiently dependent on BDNF. NT-4/5 also supports the survival of BDNF-responsive mouse nodose neurons during the phase of cell death. In the mouse, NT-4/5 appears to be just as effective as BDNF at promoting the survival of these neurons, but is a much less effective survival factor for

homologous populations of chick embryo neurons. This suggests that the structure of NT-4/5 is not well conserved between mammals and birds or that NT-4/5 is a mammalian-specific neurotrophic factor. *trk*B 'knockout' mice show significant though not great losses in trigeminal ganglion neurons and massive neuronal loss in the nodose ganglion (Klein *et al.*, 1993), consistent with the *in vitro* and *in vivo* BDNF and NT-4/5 survival requirements of these neurons (review by Davies, 1992: Davies *et al.*, 1993a).

NT-4/5 also promotes the survival of embryonic rat motoneurons *in vitro* (Henderson *et al.*, 1993). *trk*B 'knockout' mice exhibit significant motoneuron loss, especially in the facial nucleus (Klein *et al.*, 1993), while BDNF 'knockout' animals show no change in motoneuron number (Ernfors *et al.*, 1994a; Jones *et al.*, 1994). Although previous studies have shown that BDNF promotes motoneuron survival (Oppenheim, 1992; Koliatsos *et al.*, 1993; Henderson *et al.*, 1993), it appears that NT-4/5 may play a more significant role.

NT-6 Responsive Neurons

As the most recently isolated member of the NGF family of neurotrophic factors, NT-6 is the least well characterized. Recombinant fish NT-6 from a rabbit kidney cell line was found to promote the survival of embryonic chicken sensory DRG and sympathetic ganglion neurons *in vitro* but not NGF-independent ciliary and nodose neurons (Götz *et al.*, 1994). Its survival-promoting actions are similar to those of NGF but less potent.

Neurotrophic Factors Not Related To NGF

There are a number of other factors that have been shown to influence neuronal growth and survival. Important examples of these are described briefly in the following sections.

Ciliary Neurotrophic Factor (CNTF)

CNTF was first identified in and partially purified from chick eyes (Adler *et al.*, 1979) and was shown to support the survival of parasympathetic chick ciliary neurons *in vitro*. It has since been demonstrated to promote the survival of sympathetic, sensory, and spinal motoneurons in culture (Arakawa *et al.*, 1990; Barbin *et al.*,1984; Manthorpe *et al.*, 1986). More recently, a study using mice with a null mutation in the CNTF gene (Masu *et al.*, 1993) has shown that, *in vivo*, CNTF promotes the survival of motoneurons in late postnatal mice and is not important in determining motoneuron numbers during embryonic development. The cloning of CNTF has shown that it is not related to the NGF family of neurotrophic factors (Stöckli *et al.*, 1989; Lin *et al.*, 1990; Masiakowski *et al.*, 1990) but is instead a member of a family of distantly related cytokines that includes interleukin-6 (IL-6) and leukaemia inhibitory factor (LIF). Unlike NGF-related factors, it is a cytosolic protein that lacks a signal sequence for secretion (Stöckli *et al.*, 1989). Ernsberger *et al.* (1989b) have shown that CNTF blocks the division of chick sympathetic precursor cells. It also reduces tyrosine hydroxylase (TH) activity in cultures of neonatal rat sympathetic neurons while simultaneously increasing ChAT activity (Sadaat *et al.*, 1989).

CNTF binds to a multi-component receptor. The receptor complex is believed to have two sub-units in common with the receptor complex for IL-6 and one in common with the LIF receptor (Davis and Yancopoulos, 1993). The IL-6 sub-units are a glycosyl-phosphatidylinositol and a 130 kDa glycoprotein (gp130); the LIF receptor sub-unit is homologous to gp130. A third receptor component, CNTFRα, has been identified as being essential for receptor functioning (Ip *et al.*, 1993a). In CNTF-responsive cells, the binding of CNTF to its receptor induces tyrosine phosphorylation of gp130 and its homologue. The cellular mechanisms subsequent to this have yet to be elucidated.

Fibroblast Growth Factor (FGF)

The founder members of this growth factor family are basic and acidic

fibroblast growth factors (bFGF and aFGF respectively). They are cytosolic proteins of approximately 16 kDa, sharing about 55% amino acid sequence homology (Esch *et al.*, 1985). They are both found in the embryonic brain, in adult nervous tissues, and were initially identified as mitogens for fibroblasts and myoblasts (review by Gospodarowicz *et al.*, 1987). They were then shown to induce the division of endothelial cells and stimulate the differentiation and migration of these cells, indicating that FGFs may play a role in the formation of blood vessels (Folkman and Klagsbrun, 1987).

A number of studies have also demonstrated that FGF (mostly basic and acidic) can promote the survival of embryonic neurons *in vitro*. These include neurons from the hippocampus (Walicke *et al.*, 1986), the cerebral cortex, E18 rat striatum, septum, and thalamus (Walicke, 1988), and the chick ciliary ganglion and spinal cord (Unsicker *et al.*, 1987). Basic and acidic FGF have been shown to induce fibre outgrowth from several cell types, including PC12 cells (Neufeld *et al.*, 1987), newborn rat chromaffin cells (Claude *et al.*, 1988), and postnatal rat retinal ganglion cells (Lipton *et al.*, 1988).

The exact role of basic and acidic FGFs in the developing nervous system remains unclear. More recently, however, studies have been carried out using another member of the FGF family, FGF-5 (Hughes *et al.*, 1993). This was originally found to be a product of a human oncogene (Zhan *et al.*, 1987) and later identified as a member of the FGF family (Zhan *et al.*, 1988). Unlike bFGF and aFGF, it is thought to be a secretory protein (Zhan *et al.*, 1988). Hughes *et al.* (1993) have shown that recombinant human FGF-5 promotes the survival of chick embryonic spinal motoneurons *in vitro* and that both FGF-5 mRNA and protein can be detected in embryonic rat skeletal muscle during the period of naturally occurring motoneuron death as well as in adult muscle. This indicates that FGF-5 is potentially a survival factor for motoneurons during embryonic development and may play a role in maintaining them in the adult.

Glial Cell Line-Derived Neurotrophic Factor (GDNF)

GDNF was purified and cloned by Lin and co-workers (1993) from the rat B49

glial cell line and was identified as a distantly related member of the transforming growth factor-β (TGF-β) family (review by Massagué *et al.*, 1994). It comprises a glycosylated, disulphide-bonded homodimer. Human and rat GDNF genes have also been cloned (Lin *et al.*, 1993).

Lin *et al.* (1993) have studied the effect of GDNF on dopaminergic neurons in dissociated cultures of embryonic rat midbrain. They used high-affinity dopamine uptake and expression of TH as markers for dopaminergic neuron survival and differentiation. GDNF promotes the survival of TH^+ dopaminergic neurons, enhances their dopamine uptake, and is expressed in the striatum, their target field. More recently, studies have shown that GDNF protects adult dopaminergic neurons from axotomy-induced degeneration *in vivo* (Tomac *et al.*, 1995; Beck *et al.*, 1995).

Henderson *et al.* (1994) have studied the effects of GDNF on motoneuron survival *in vitro* and *in vivo*. They found that GDNF promotes the survival of embryonic motoneurons *in vitro*, being more potent than BDNF, NT-3, NT-4/5, and CNTF. *In vivo*, it rescues facial motoneurons from axotomy-induced cell death. Henderson and co-workers (1994) have also detected GDNF mRNA in the vicinity of motoneurons during the period of naturally occurring cell death. More recently, similar studies by Yan *et al.* (1995), using neonatal and adult facial motoneurons, and by Oppenheim *et al.* (1995), using developing facial motoneurons, have verified the importance of GDNF in the survival of these neurons. This is strong evidence that GDNF is a physiological survival factor for motoneurons.

Aims of Project

Much of the evidence for the neurotrophic hypothesis has come from work on NGF. The main aim of the research project was to explore the generality of the neurotrophic hypothesis but focusing on the second member of the neurotrophin family.

In the initial study, I have attempted to clarify whether the low-affinity p75 receptor is important in the functioning of one particular neurotrophin. p75 mRNA

levels were measured in purified populations of embryonic chicken neurons that require different neurotrophic factors for survival to establish whether these levels were significantly higher in NGF- or BDNF-responsive neurons. I have also studied the effects of various factors, for example, NGF, CNTF, and membrane depolarization, on neuronal p75 mRNA expression *in vitro*.

BDNF mRNA is expressed in a proportion of embryonic DRG neurons which raises the possibility that BDNF acts by an autocrine loop in these neurons. The aim of the second study was to investigate the role of BDNF expression in developing sensory neurons, particularly during the phase of naturally occurring death when the neurons are dependent on target-derived neurotrophins for survival. I have compared the levels of BDNF mRNA in purified populations of embryonic chicken cranial sensory neurons that depend on either NGF or BDNF for survival. A co-culture technique was then used to show whether a paracrine survival mechanism, involving the production and release of BDNF, might exist between NGF- and BDNF-dependent neurons.

The first detailed studies of the timing and regulation of *trk*B and BDNF mRNA expression have been carried out using two populations of sensory neurons that innervate their targets at different stages of development. In the chicken, neurons of the vestibular and nodose ganglia are born during the same stage of development and start extending axons to their targets at about the same time, but the distance their axons have to grow to reach their targets differs greatly. In the final study, I have related the timing of *trk*B mRNA expression in these neurons to the timing of target field innervation and have studied the expression of BDNF mRNA in these targets at stages before their innervation. I have also studied the effect of BDNF on the regulation of *trk*B mRNA expression in BDNF-independent early nodose neurons *in vitro*. The timing and regulation of expression of BDNF and *trk*B mRNAs shows a number of similarities with NGF and *trk*A expression and some important differences.

CHAPTER 2

Materials and Methods

The normal expression and *in vitro* regulation of expression of p75, BDNF, and *trk*B mRNAs were determined for a variety of embryonic chicken sensory and autonomic neurons using a highly sensitive reverse transcription-polymerase chain reaction (RT-PCR) assay. This technique was chosen in favour of Northern blotting as it allowed for quantitation of these rare mRNAs from relatively few neurons (50-80 neurons in the case of BDNF mRNA). Where neuronal number could not be accurately obtained, the p75, BDNF and *trk*B mRNA levels were standardized with respect to the level of mRNA encoding the ubiquitous and constitutively expressed ribosomal protein L27. The L27 mRNA level was measured in a separate PCR. A range of molecular biology techniques was also used to subclone p75, BDNF, *trk*B, and L27 cDNA fragments and so prepare the plasmid constructs from which cRNA standards added to the RT-PCR assays were synthesized. Both these and the RT-PCR assay are described below, along with details of how the various embryonic neurons were obtained through dissection, separated from non-neuronal cells, and, in certain cases, set up in culture. Unless otherwise stated, the reagents used were purchased from BDH or Sigma. Oligonucleotide primers were either purchased from British Biotech or synthesized using an Applied Biosystems PCR Mate.

Molecular Biology Techniques

Subcloning of cDNA Fragments

Plasmid Vectors

p75, BDNF, *trk*B, and L27 cDNA fragments were more easily manipulated by inserting them into circular plasmid or vector DNA, producing a recombinant plasmid. The vectors used in this study were pGem-3Z and -4Z, supplied by Promega. They contain identical multiple cloning regions bearing a versatile selection of sites

recognised by restriction endonucleases, into which DNA fragments can be inserted. Once in the vector, the cDNA fragment can be amplified in the appropriate host bacterial strain and, after plasmid isolation, sequenced and modified as desired. The vectors are differentiated by the orientation of their multiple cloning regions. SP6 and T7 RNA polymerase promoter sites also flank the multiple cloning regions such that RNA can be transcribed from either strand of the plasmids, although, for the purpose of this study, only the sense transcript was required. Other properties of the pGem vectors are discussed in more detail in later sections.

Digestion of DNA by Restriction Endonucleases

The subcloning procedure involved the initial digestion of cDNA and vector with appropriate restriction endonucleases to generate compatible ends for ligation. In this study, the production of constructs for the cRNA RT-PCR controls involved the excision of the desired cDNA fragment from a larger piece of DNA in a vector or the digestion of a PCR product, prior to insertion into the appropriate vector (individual cases will be discussed in more detail in the section on RT-PCR).

5-10 µg of DNA were digested in a 50 µl reaction containing 5 µl 10x restriction endonuclease buffer (usually supplied with the enzyme), the restriction endonuclease itself, and sufficient sterile, deionized water to give the desired volume. 1-5 µg of DNA were digested in a 20 µl reaction. The reactions were carried out at the recommended optimum temperature for each endonuclease (usually 37°C).

In general, restriction endonucleases were purchased from Gibco BRL and supplied in concentrations ranging from 5-50 units/µl, where 1 unit represents the amount of enzyme required to digest 1 µg of substrate DNA in 60 minutes under the recommended assay conditions. The reactions were carried out with a five- to ten- fold excess of endonuclease for 1-2 hours. Longer incubations were avoided as contaminating exonucleases in the endonuclease preparation may digest away the cleaved endonuclease recognition sites and so affect subsequent ligation procedures.

Digestion of DNA with two restriction endonucleases was carried out

simultaneously when both enzymes required the same optimal conditions. However, when the enzymes' optimal conditions were not compatible, the DNA was first incubated with the enzyme requiring the buffer with the lowest salt concentration. The reaction mixture was adjusted to meet the buffering requirements of the second enzyme which was then added and the incubation allowed to proceed. The efficiency of the digests was determined using electrophoresis by visualizing 0.5 μg on a 1% agarose/Tris-acetate-EDTA (TAE) gel. This procedure is described after this section.

If the vector was digested with a single restriction enzyme, it was treated with calf intestinal alkaline phosphatase (CIAP) to remove 5' phosphate groups and so prevent the vector from re-circularizing without insert during the ligation reaction. For CIAP treatment, the following were added to 50 μl of the vector digest (containing 5 μg DNA): 10x CIAP buffer (500 mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 10 mM spermidine), 1 μl CIAP, and sterile, deionized water to a final volume of 100 μl. The reaction was incubated at 37°C for 1 hour.

The CIAP was removed by addition of one volume of tris-buffered phenol (pH 8.0) and, after mixing, an equal volume of chloroform/isoamyl alcohol (24:1). After a further mixing and centrifugation (2 minutes at 13,000 rpm), the upper aqueous phase was removed to a fresh Eppendorf tube. The DNA was precipitated by addition of 1/9th volume of 5 M sodium acetate, pH 5.0, and 2.5 volumes of absolute ethanol, and pelleted by centrifugation (13,000 rpm) for 1 minute. The supernatant was removed and the pellet washed free of salts with 1 ml 70% ethanol. After a further brief centrifugation, the supernatant was removed and the pellet air-dried and resuspended in 50 μl of sterile, deionized water. The quantity of DNA recovered was estimated by visualizing 5 μl of the resuspended DNA on a 1% agarose/TAE gel.

Purification of Digested Insert and Vector Fragments

Prior to ligation of vector and insert, the relevant DNA fragments were first isolated and purified from the digests. Digested vector and insert fragments were separated by agarose/TAE gel electrophoresis. The Geneclean II kit (BIO 101, Stratech)

was used to purify the fragments from the gel. The desired DNA bands were excised from the gel and cut into small pieces. 3 ml of NaI (from the kit) were added per gram of agarose and the NaI/agarose mixture placed at 55°C until the agarose was completely melted. 5-10 µl of Geneclean Glassmilk were then added to the mixture and this left, with intermittent mixing, at room temperature for 10 minutes to allow the the DNA to bind.

The Glassmilk and bound DNA were pelleted by brief centrifugation at 13,000 rpm and the supernatant removed. The Glassmilk was washed by the addition of 1 ml of the supplied New Wash solution, followed by resuspension and re-pelleting by brief centrifugation (at 13,000 rpm). The supernatant was removed and the washing procedure repeated one further time. After the final centrifugation, as much of the New Wash as possible was removed using a pipette with a fine-bore tip and the DNA eluted at 55°C into 10-20 µl of sterile, deionized water for 10-30 minutes. The Glassmilk was pelleted by centrifugation (at 13,000 rpm) for 1 minute and the solution of eluted DNA removed to a fresh Eppendorf tube. The purified fragments were visualized by loading 2-5 µl on a 1% agarose/TAE gel. The approximate amount of each fragment was estimated from the gel.

Ligation of DNA Fragment and Vector

Insertion of a DNA fragment into the vector was achieved by means of the ligation reaction. This utilized the enzyme bacteriophage T4 DNA ligase (a single polypeptide of M_r = 68,000) which catalyzes the formation of a phosphodiester bond between adjacent 3'-OH and 5'-P termini of double-stranded DNA.

Prior to setting up the ligation, it was necessary to optimize the ratio of vector to insert with respect to molecule number. Where the ligation was directional, with both vector and insert having two different termini, this ratio was 1:1. When the insert DNA was ligated into a single restriction site in the (CIAP-treated) vector, the optimum vector:insert ratio was 1:3. This ratio was estimated by visualizing equal volumes of vector and insert on an agarose/TAE gel and comparing the intensity of ethidium

bromide staining with that of a DNA sample of known concentration. Once the amounts of vector and insert were determined, it was then necessary to take into account their size differences so that the vector:insert ratio was based on the relative number of molecules. Between 100-200 ng of vector DNA were added to the ligation and the amount of insert DNA added was determined in accordance with this.

The appropriate volumes of vector and DNA insert were added to a ligation reaction containing 1 µl T4 DNA ligase (Northumbria Biologicals), 1 µl 10x ligation buffer (supplied with the enzyme), and sterile, deionized water to 10 µl. The reaction was then incubated at 15°C for at least 4 hours. This procedure was suitable for the ligation of DNA fragments with cohesive termini. Where the termini were blunt-ended, however, it was found that the addition of 1 mM hexamminecobalt (III) chloride to the reaction mixture increased the efficiency of ligation. This complex ion increases the effective concentration of insert and vector DNA. Control ligation reactions were also set up containing vector only.

At this stage, the amount of ligated vector plus insert (or recombinant plasmid) obtained was insufficient for subsequent procedures. The usual way of increasing the quantity of plasmid DNA is to facilitate its uptake by host bacteria in which it is then replicated as the bacteria propagate. This procedure (referred to as transformation) is described in the following sections.

Bacterial Host Strain

The *Escherichia coli* (*E. coli*) host strain of XL1 Blue was used in the amplification of all plasmid constructs from which the RT-PCR cRNA controls were prepared. The pGem vectors used bear the β lactamase gene which confers ampicillin resistance to the host cells. This allows for the selection of transformed cells in culture and when plated out. XL1 Blue has the genotype *sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi rel*A1 *lac*$^-$ F'[*pro*AB$^+$ *lac*^{q} *lac*Z ΔM15 Tn10 (*tet*^{r})]. *end*A1 is an endonuclease mutation and improves the quality of plasmid DNA isolations. The *lac*Z mutation is a partial deletion of the β-D-galactosidase gene. This gene is activated by the pGem

vector α-complementation sequence in nonrecombinant plasmids. In recombinant plasmids, this sequence is interrupted by the presence of insert DNA and α-complementation is not possible. This feature allows for colour selection between bacterial colonies bearing nonrecombinant and recombinant plasmids, when the cells are plated out on agar supplemented with the substrate for β-D-galactosidase (i.e., X-gal and IPTG).

Preparation of Competent Host Cells and Transformation

The following method for preparing competent cells gave a transformation frequency of 10^6 transformant colonies/μg of supercoiled DNA, which was sufficient for the cDNA subcloning carried out in this study.

As far as possible, all manual procedures were carried out in a laminar flow cabinet to maintain sterility. Stocks of XL1 Blue cells were kept at -70°C in Luria broth (L broth)/15% glycerol. XL1 Blue cells were scraped from the surface of a frozen stock aliquot and streaked across Luria agar (L agar) using a flamed inoculating loop, so as to produce single colonies after an incubation of 12-16 hours at 37°C. The dish was incubated in an inverted position. L agar and L broth powders were purchased from Gibco BRL.

A culture was prepared by using a flamed loop to inoculate 25ml of sterile L broth from a single colony from the petri dish, and this was incubated overnight at 37°C in a rotary incubator. The 25 ml overnight culture was then diluted in 250 ml of sterile L broth to give an optical density at 550 nm (OD_{550}) of at least 0.0015. The diluted culture was then grown at 37°C in a rotary incubator until the OD_{550} was 0.48, or just below. It was then chilled on ice for 5 minutes and the bacterial cells pelleted by centrifuging in sterile, ice-cold tubes at 6,000 rpm (10 minutes, 4°C). From this point onwards, it was essential to keep the cells as cold as possible by placing them on ice whenever possible. The supernatant broth was discarded and the cells were gently resuspended in 100 ml of filter-sterilized, ice-cold buffer I (30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride, and

15% glycerol, pH 5.8). The cells were left on ice in buffer I for 5 minutes and pelleted at 6,000 rpm (5 minutes at 4°C). The supernatant was discarded and the cells resuspended in 10 ml of filter-sterilized, ice-cold buffer II (10 mM MOPS, 75 mM calcium chloride, 10 mM rubidium chloride, and 15% glycerol, pH 6.5). The cells were left on ice in buffer II for 15 minutes. They were then aliquoted in 200 µl volumes into sterile Eppendorf tubes placed in a mixture of dry ice and IMS to 'snap' freeze them, and stored at -70°C.

The competent cells were transformed with plasmid of known concentration to test their efficiency using the following procedure. This transformation was usually carried out using 1-2 ng of DNA which should give a transformed cell number in the range of 10^3. One Eppendorf tube of competent cells was first removed from storage at -70°C and placed directly on ice to defrost. It was essential that the cells were not left at room temperature as they would lose their competency. The plasmid DNA was also pre-cooled on ice. Once defrosted, the cells were gently mixed and 100 µl were removed to a fresh Eppendorf tube. 1-2 ng of DNA were then added to one 100 µl aliquot of cells (the volume of DNA added should not exceed 10 µl) and this was left on ice for 30 minutes. The other100 µl aliquot of cells was left on ice without any added DNA and would be put through the transformation procedure to check whether the cells had been contaminated with DNA during preparation. The cells were then heat shocked at 42°C for 2 minutes and returned directly to ice. 500 µl of sterile L broth (without antibiotic) were added to each tube and the cells were incubated at 37°C in a rotary incubator for 30 minutes, to allow the transformed cells to start expressing the plasmid-borne antibiotic resistance gene. The cells were then plated onto L agar containing the antibiotic selecting for transformed cells. In the case of the pGem vectors, it was ampicillin. The ampicillin was prepared as a 1000x stock solution of 100 mg/ml in deionized water. It was added to molten agar cooled to 55°C so that it was not deactivated. 200 µl of undiluted transformed cells were plated out using a sterile glass spreader first immersed in alcohol and then flamed. 200 µl of a 1 in 10 and a 1 in 100 dilution (in L broth) of the transformed cells were also plated in case the

number of colonies from the undiluted transformation mixture was too numerous to be counted. 200 µl of the control cells (with no DNA added) were also plated out. The surfaces of the petri dishes were left to dry and the dishes then incubated inverted for 12-16 hours at 37°C.

The transformation procedure using ligation and control (vector only) ligation mixtures was essentially the same [100 µl of competent cells were transformed with all 10 µl of the ligation mixture (containing nanogram levels of ligated plasmid DNA) and this treated as described above]. 50, 100, and 200 µl of the transformed cells were plated out on L agar with 100 µg/ml ampicillin to select for transformants. X-gal and IPTG may also be added to the agar to allow for colour selection of recombinant from nonrecombinant plasmids. The plates were incubated inverted at 37°C for 12-16 hours.

It was then necessary to determine whether the transformed colonies obtained bore the desired recombinant plasmid. A crude, small-scale plasmid isolation procedure was adequate for this purpose. Single colonies were used to inoculate several 5 ml aliquots of sterile L broth (plus 100 µg/ml ampicillin) and these placed in a rotary incubator at 37°C for 12-16 hours. Plasmid DNA isolation was then carried out as described below.

Small-Scale Isolation of Plasmid DNA by Alkaline Lysis

The plasmid 'mini-prep' procedure used in this study allowed for the quick and convenient isolation of plasmid DNA without column purification or banding in caesium chloride gradients by ultracentrifugation. It made use of the rapid alkaline denaturation of plasmid and chromosomal DNA and the selective renaturation of plasmid DNA after neutralization of the solution. The resultant plasmid DNA was suitable for restriction enzyme analysis. The procedure could be scaled-up and modified to include additional ammonium acetate and polyethylene glycol purificaton steps, giving a greater quantity of DNA suitable for restriction enzyme digestion and subcloning, sequencing, and *in vitro* transcription. This protocol is described in a later section.

1 ml samples of the overnight cultures prepared from transformed colonies were centrifuged (at 13,000 rpm) in Eppendorf tubes to pellet the bacterial cells. The pGem plasmids are high copy number plasmids, with approximately 300-400 copies/cell; 1 ml of culture yielded 1-4 μg of DNA.

The supernatant was discarded and the bacterial pellet resuspended in 100 μl of Solution I (50 mM glucose, 25 mM Tris at pH 8.0, 10 mM EDTA). The tubes were placed on ice, 200 μl of denaturing Solution II (0.2 M sodium hydroxide, 1% SDS) added, and this gently mixed. 150 μl of Solution III (per 100 ml: 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, 28.5 ml of water-pH 4.8) were then added immediately to neutralize Solution II.

Cell debris and chromosomal DNA were pelleted by centrifugation at 13,000 rpm for 1 minute. The supernatant was transferred to a fresh Eppendorf tube and the plasmid DNA precipitated by the addition of 1 ml of absolute ethanol. After mixing, the plasmid DNA was pelleted by centrifugation at 13,000 rpm for 1 minute, the supernatant removed, and the pellet air-dried. The DNA was resuspended in 50 μl of sterile, deionized water.

RNA in the DNA preparation was degraded by treatment with 1 μl of 10 mg/ml bovine pancreatic ribonuclease (RNase) from Pharmacia. This was added at room temperature and the DNA solution thoroughly mixed; no incubation time was necessary. The DNA solution was deproteinated by phenol-chloroform extraction. 50 μl of tris-equilibrated phenol were added to denature the protein and the solution was mixed well. 50 μl chloroform/isoamyl alcohol (24:1) were then added and the solution mixed again to extract the phenol plus denatured protein in the chloroform. The mixture was centrifuged at 13,000 rpm for 3 minutes to separate aqueous and non-aqueous phases. The upper aqueous phase containing the DNA was transferred to a fresh Eppendorf tube. The DNA was precipitated by the addition of 1/9th volume of 3 M sodium acetate, pH 5.2 and 2.5 volumes of absolute ethanol. After mixing and centrifugation at 13,000 rpm for 1 minute, the supernatant was removed and the DNA pellet washed free of salts with 1 ml of 70% ethanol. The ethanol wash solution was

removed, and the DNA pellet air-dried and resuspended in 50 µl of sterile, deionized water. 10 µl of the 'mini-prep' could then be visualized on an agarose/TAE gel and analyzed using restriction enzymes. 'Mini-prep' DNA is best stored at -20°C or lower as it is prone to degradation by contaminating nucleases.

Once it has been ascertained that the isolated plasmid DNA is the desired one, a few aliquots of the original overnight culture were mixed with 15% glycerol and stored at -70°C. A purer, larger-scale plasmid DNA preparation was then carried out for future procedures.

Isolation of Plasmid DNA by Alkaline Lysis-Polyethylene Glycol

For the purpose of this study, isolation of plasmid DNA by alkaline lysis with subsequent purification using polyethylene glycol (PEG) was adequate. The DNA obtained could be used for sequencing, subcloning procedures, and *in vitro* transcription. The technique was also flexible in that it could be easily scaled-up depending on the yield of plasmid DNA required. The yield given by a method employing a caesium chloride gradient would have far exceeded the requirements of this study, not to mention the extra expense and time it would require. The PEG method utilizes the same Solutions I, II, and III as the small-scale DNA isolation procedure.

The appropriate 'mini-prep' overnight culture was streaked onto L agar with 100 µg/ml ampicillin. The plate was incubated at 37°C so as to produce single colonies. A single colony was used to inoculate 10 ml L broth with 100 µg/ml ampicillin. The culture was then grown up at 37°C in a rotary incubator for 12-16 hours.

The bacterial cells were pelleted by centrifugation at 6,000 rpm for 10 minutes at 2°C. The supernatant was discarded and the pellet resuspended in 400 µl of Solution I. If desired, a 'pinch' of lysozyme could be added at this point and the mixture left for a few minutes. 800 µl of Solution II were then added to denature the DNA and this mixed gently. 600 µl of Solution III were then added immediately to neutralize the

mixture.

Cell debris and chromosomal DNA were then pelleted by centrifugation at 11,500 rpm for 20 minutes at 0°C. The supernatant was then removed to two Eppendorf tubes (approximately 900 µl/tube). 500 µl of isopropanol were added to each tube to precipitate the DNA, and this mixed and left at -20°C for 15 minutes. The DNA was pelleted in a refrigerated centrifuge at 13,000 rpm for 5 minutes. The pellets were washed in 1 ml of 70% ethanol, air-dried, and each resuspended in 50 µl of sterile, deionized water.

RNA and denatured plasmid DNA were precipitated by addition of 100 µl of 7.5 M ammonium acetate to each tube. This was mixed and left at -20°C for 15 minutes. The RNA and denatured plasmid DNA were pelleted in a refrigerated centrifuge at 13,000 rpm for 5 minutes. The supernatants from each tube were removed and pooled in a fresh Eppendorf tube, and the DNA precipitated by the addition of 0.6 volume of isopropanol. The DNA was pelleted in a refrigerated centrifuge at 13,000 rpm for 5 minutes. The pellet was washed with 1 ml of 70% ethanol, air dried, and resuspended in 500 µl of sterile, deionized water.

The DNA was then further purified with polyethylene glycol. 500 µl of a solution containing 11.2% (w/v) PEG 6000/8000 and 0.7 M sodium chloride were added to the resuspended DNA, this mixed and left on ice for 30 minutes. RNA and denatured DNA not precipitated by the ammonium acetate were then pelleted in a refrigerated centrifuge at 13,000 rpm for 5 minutes. The supernatant was then transferred to a fresh Eppendorf tube and a further 500 µl of the PEG/NaCl solution added. This was mixed and left on ice for 30 minutes. Increasing the concentration of PEG precipitated the plasmid DNA and this was pelleted in a refrigerated centrifuge at 13,000 rpm for 5 minutes. The pellet was washed three times in 1 ml 70% ethanol to ensure that all the PEG was removed. It was then air dried and resuspended in 50 µl of sterile, deionized water.

The yield of plasmid DNA obtained from this procedure was usually in the range of 0.5-1.0 mg/ml. The concentration of DNA in the sample was determined by

ultraviolet (UV) spectroscopy. This method is reliable providing the sample is pure (that is, free from significant amounts of UV-absorbing protein or RNA). OD readings were taken at 260 nm and 280 nm. The OD_{260} reading was used to calculate the DNA concentration; an OD_{260} of 1 represents approximately 50 μg/ml for double-stranded DNA. The ratio of OD_{260} to OD_{280} (OD_{260}/OD_{280}) gives an indication of the purity of the DNA sample. A good DNA preparation would give an OD_{260}/OD_{280} of between 1.75-1.85, although, ideally, it should be as close to 1.8 as possible. Values greater than 1.8 indicate that the sample is contaminated with RNA, while lower values indicate protein. As it was difficult to determine the effect of contamination on the OD readings, the concentration was also determined by estimating the intensity of fluorescence emitted by the ethidium bromide-stained DNA after running 1 μl of the sample on an agarose gel, alongside a standard DNA sample of known concentration. (Sambrook *et al.*, 1989).

Once again the plasmid DNA is best stored at temperatures of -20°C or lower, although short-term storage at 4°C would not be detrimental as nuclease contamination is negligible in this case.

Agarose Gel Electrophoresis of DNA

DNA fragments were separated by electrophoresis through agarose gels. This relies on the principle that DNA, which is negatively-charged at neutral pH, will migrate from cathode to anode when a potential difference is applied across the gel. The rate of migration of linear, duplex DNA molecules is inversely proportional to the $\log_{10}$ of their molecular weights. It is also affected by the agarose concentration of the gel, the DNA conformation (i.e., whether it is linear, circular, or supercoiled), the voltage applied across the gel, and the composition and ionic strength of the electrophoresis buffer. 1-2% agarose gels were sufficient to separate fragments 0.1-5 kb in size.

Tris-acetate (TAE) electrophoresis buffer was used for agarose gel electrophoresis. It was prepared as a 50x stock solution comprising 242 g Tris base, 57.1 ml glacial acetic acid 100 ml 0.5 M EDTA (pH 8.0), and deionized water to 1 litre.

For a Pharmacia Minigel, the TAE stock solution was diluted to give 500 ml of 1x TAE, of which 50 ml were used for gel preparation. The desired amount of ultra pure nuclease-free agarose (Gibco BRL) was completely melted in the TAE and the intercalating dye ethidium bromide added to a final concentration of 0.50 μg/ml to both molten gel and the remainder of the buffer. The ethidium bromide binds to the DNA during electrophoresis. It fluoresces in ultraviolet (UV) light, allowing DNA bands on a gel to be viewed effectively. Once the molten gel had cooled to 60°C, it was carefully poured into a gel-casting plate sealed at either end with masking tape, and the well-forming plastic or comb placed in position. The gel was left to set for 20-30 minutes.

The set gel was placed in the electrophoresis tank and covered with TAE buffer. The comb was carefully removed and more buffer added to ensure that the wells were covered and free from air bubbles. The DNA samples were mixed with the appropriate volume of 6x gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol in deionized water) and loaded onto the gel. DNA size markers, containing DNA fragments of known lengths, were also loaded onto the gel to facilitate identification of the sample DNA.

A voltage of 1-5 V/cm (measured as the distance between the electrodes) was applied across the gel. At higher voltages, the migration rate of high-molecular-weight DNA increases differentially, so reducing the resolution properties of the gel. The duration of electrophoresis depended on DNA fragment size and the degree of separation required. The progress of fragment migration could be monitored by occasional viewing on a UV transilluminator. If desired, a permanent record of the gel could be made by photographing it on the transilluminator after electrophoresis was complete.

Sequencing of Double-Stranded DNA

DNA sequencing was used to check the identity of *trk*B and L27 cDNA obtained through reverse transcription and polymerase chain reaction, after subcloning into pGem-3Z, and to check modifications made to p75, BDNF, *trk*B, and L27 cDNAs

during preparation of the PCR control templates.

The method of sequencing used was that of dideoxy chain termination (Sanger *et al.*, 1977). This involves a DNA polymerase elongating a DNA strand from a specific primer annealed to a single-stranded DNA template. Also included in the reaction are a mixture of the four nucleotides (dNTPs), ^{35}S (or ^{32}P) labelled dATP, and, subsequently, one of four modified nucleotides. These modified bases are 2',3'-dideoxynucleoside 5'-triphosphates (ddNTPs) which terminate DNA elongation; they lack the deoxyribose 3' hydroxyl group necessary for the formation of a phosphodiester bond with a succeeding nucleotide. In this sequencing reaction, there are both elongation and, less frequently, specific termination of the DNA strand. The reaction products are a series of oligonucleotides, the lengths of which are determined by the distance between primer and termination sites. The four different ddNTPs are added to four separate reactions and populations of oligonucleotides are synthesized that terminate at positions occupied by every A, C, G, or T in the template strand. The reaction products are separated on a polyacrylamide gel, which is then dried down and placed in contact with X-ray film. The resultant autoradiograph bears a ladder of bands representing DNA strands of different lengths, for each of the four synthesis reactions. Taking all four reactions into consideration, the order of the bands, from bottom to top of the autoradiograph, gives the sequence of the template.

In this case, the sequencing primers used were synthetic 17mers complementary to sequences in the pGem vectors just upstream (3') from the T7 RNA polymerase promoter (T7 primer) and just downstream (5') from the SP6 RNA polymerase promoter (SP6 primer).

The quality of double-stranded DNA templates is of great importance. They should be purified and RNA-free. Purification using PEG precipitation (as described previously) was found to be effective. The choice of DNA polymerase used is also critical. Sequenase Version 2.0 (United States Biochemical) was found to very reliable. This enzyme is a genetically modified variant of bacteriophage T7 DNA polymerase, in which the 3'-5' exonuclease activity of the wild-type T7 DNA polymerase has been

removed. The enzyme is both stable and efficient.

The double-stranded DNA was first alkaline denatured as follows. To 8 μg of PEG purified DNA, 1/10th volume of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol were added to precipitate the DNA. After brief centrifugation (13,000 rpm), the resultant DNA pellet was washed in 70% ethanol, re-pelleted, and air dried. The pellet was resuspended in 40 μl of 0.2 M NaOH/0.2 mM EDTA (pH 8.0) and left to denature at room temperature for 5 minutes. 4 μl of 3 M sodium acetate (pH 5.2) were then added to neutralize the solution and the single-stranded DNA precipitated by the addition of 2 volumes of absolute ethanol. The precipitated DNA could be left overnight at -20°C if desired, but this was not essential. The DNA was pelleted by centrifugation at 13,000 rpm for 3 minutes, washed in 70% ethanol, re-pelleted, and air dried. The pellet was then resuspended in 14 μl of sterile, deionized water.

7 μl of the resuspended single-stranded DNA were used for each sequencing primer. The next step in sequencing involved the annealing of primer to template. To 7 μl of the single-stranded DNA were added 1 μl of primer (approximately 50 ng) and 2 μl of Sequenase Reaction Buffer (from the Sequenase Version 2.0 Sequencing Kit). This was incubated at 37°C for 15 minutes, to allow the primer to anneal to the template DNA.

The Sequenase protocol then divided the sequencing procedure into two stages. An initial synthesis at low temperature utilising low dNTP concentrations (i.e., the labelling reaction) allowed for sufficient incorporation of ^{35}S labelled dATP. The labelling reaction was then divided into four termination reactions, each containing higher concentrations of the conventional dNTPs and one of the four ddNTPs.

The components of the labelling reaction were added on ice directly to the 10 μl annealing reaction. These included the dGTP Labelling Mix and Sequenase enzyme. The dGTP Labelling Mix (from the Sequenase Version 2.0 kit), comprising a mixture of all four dNTPs, was diluted 1 in 5 with sterile, deionized water, or 1 in 10 if sequences close to the primer were desired. The Sequenase enzyme itself was also diluted 1 in 8 before use with cold Enzyme Dilution Buffer (from the kit) and was

stored on ice for not more than 1 hour. Thus, the following components of the labelling reaction were added to the 10 µl annealing reaction: 1 µl DTT (from the kit), 2 µl dilute Labelling Mix, 0.5 µl 10 µCi/µl ^{35}S labelled dATP, and 2 µl diluted Sequenase enzyme. The reactants were mixed carefully to avoid air bubbles and incubated at room temperature for 5-10 minutes, depending on how close to the primer sequences needed to be read. If sequences close to the primer were desired, this incubation step was omitted.

0.5 ml Eppendorf tubes containing 2.5 µl ddGTP, ddCTP, ddATP, and ddTTP were prepared and pre-warmed to 37°C 1 minute before use. 3.5 µl of the labelling reaction were added to each Eppendorf tube at 37°C. After careful mixing in the pipette tip, the termination reactions were allowed to proceed at 37°C for 5 minutes. The termination reactions were stopped by the addition of 4 µl Stop Solution (from the kit). The reactions were stored on ice or at -20°C until electrophoresis.

Polyacrylamide Gel Electrophoresis of Sequencing Products

Sequencing products were separated using denaturing gels containing 7 M urea and 6% polyacrylamide. The gel apparatus used was purchased from Flowgen and comprised 21 x 50 cm glass plates with 0.4 mm spacers and comb. The apparatus was assembled according to the manufacturer's instructions. Prior to assembly, it was ensured that one plate was washed thoroughly and cleansed free of grease with 70% IMS, while the other plate was washed and siliconized. This enabled the plates to be separated after electrophoresis such that the gel was attached to the non-siliconized plate.

The sequencing gel solution contained 7 M urea, 6% acrylamide/bis (19:1), and 1x TBE (5x TBE comprises 54 g Tris base, 27.5 g boric acid, 20 ml 0.5 M EDTA at pH 8.0, and deionized water to 1 litre) and was prepared as required. The acrylamide/bis solution was stored at 4°C as a 30% stock. During preparation of the gel solution, the acrylamide and dissolved urea were deionized using Amberlite MB-1 Mixed Bed Resin (BDH). The gel was cast as follows. 135 µl of TEMED and 800 µl

of freshly-made 10% ammonium persulphate were added to 90 ml of gel solution. This was mixed and taken up into a 60 ml syringe. With the assembled gel plates held at angle of approximately 30° to the horizontal, the gel solution was carefully introduced into the space between the plates, taking care to avoid air bubbles. Once this space was completely filled, a comb was inserted at the top of the gel, which was left to polymerize for at least 30-45 minutes.

The polymerized gel was placed in the gel apparatus, the buffer reservoirs of which were then filled with 1x TBE. The comb was carefully removed from the gel and unpolymerized acrylamide flushed from the wells with buffer using a pasteur pipette. The gel apparatus was connected to a high-voltage supply and the gel pre-run without loaded samples at 1,300-1,500 volts until the temperature of the gel reached approximately 50°C (as shown by a temperature indicator placed on the front of the gel plate).

Once the temperature of the gel had reached 50°C, the power supply was disconnected and the wells flushed out as before to remove any urea that had leached out of the gel. Prior to loading, the sequencing samples were denatured by heating to 75-80°C for 2 minutes and then placed directly on ice. 2-3 µl of each sample were loaded into the wells using a fine-bore pipette and 'duck-billed' tips. To ensure good resolution, the samples were loaded as quickly as possible. Hence, in situations where a large number of samples were being run, the loading procedure was staggered.

Once the samples were loaded, the power supply was reconnected and adjusted to 1,500 volts. During electrophoresis, the voltage was adjusted periodically so that the temperature of the gel was kept at approximately 50°C for optimum resolution of the samples. At temperatures higher than 55°C, acrylamide/urea gels may hydrolyze, resulting in loss of resolution. The duration of electrophoresis was dependent on the length of sequence required.

After completion of electrophoresis, the power supply was disconnected and the glass plate/gel assembly was removed from the apparatus. The glass plates were carefully separated, leaving the gel attached to the non-siliconized plate. The plate with

gel attached was immersed in a solution of 10% acetic acid/12% methanol for 30 minutes to remove urea from the gel as this dampens down ^{35}S emissions. The gel was taken up onto a piece of Whatman 3MM Paper, covered with Saran Wrap, and vacuum dried on a slab gel drier at 80°C. The Saran Wrap was removed from the gel, which was then placed in a light-proof cassette in contact with X-ray film. In general, an overnight exposure was sufficient to visualize the sequencing reaction products.

Reverse Transcription-Polymerase Chain Reaction Assay

The reverse transcription-polymerase chain (RT-PCR) assay used in this study was designed to measure very low mRNA levels. It involved the initial reverse transcription of extracted total RNA and the subsequent PCR amplification of the resultant cDNA using specific primers. As the efficiency of individual PCRs was affected by factors such as the composition of the reaction mix, differences in temperature across the heating block, etc., it was necessary to include an internal PCR control template of known concentration which amplified with the same primers and to which the level of native template could be directly related. Also, the use of 'master mixes' when setting up batches of PCRs was found to provide some uniformity in reaction conditions.

The controls were prepared from p75, BDNF, and L27 cDNAs so as to be 4 bp larger than the native template and from *trk*B cDNA so as to be 8 bp larger. This allowed for electrophoretic separation of the two PCR species. Primer sites were chosen to give small amplified products (for the native templates, 88 bp (p75), 100 bp (BDNF), 98 bp (*trk*B), and 127 bp (L27)). For this size range of amplified product, a 4-8 bp difference between native and control templates was sufficient for separating the two species on a 7% polyacrylamide gel. It was important that the size difference be kept to a minimum to ensure that control and native templates were almost identical and so amplified with near equal efficiency. The efficiency of PCR amplification of a cDNA species is dependent on the kinetics of primer-template annealing, the length of template being amplified, and the sequence and secondary structure of the template. Therefore,

reaction conditions may not equally favour the amplification of structurally unrelated templates.

Controls were added as cRNA to the reverse transcription so minimizing any discrepancies at this stage. The amount of target mRNA in the sample was estimated from the PCR product by calculating its ratio to cRNA control of known concentration. More accurate values could be determined by titrating the RNA sample against different concentrations of control template. The ratio between control template and target mRNA is only quantitative while the PCR is in the logarithmic phase. At this stage, the PCR products were not sufficient to be visualized by ethidium bromide staining. Radiolabelling the product was a more sensitive means of detection. This was achieved by labelling the PCR primers to a high specific activity on their 5' ends with ^{32}P ATP using T4 polynucleotide kinase. This enabled small amounts of target cDNA (0.5-1 fg) in the reaction to be visualized, after autoradiographic exposure times of a few hours, using only a few rounds of amplification.

A small PCR product was advantageous in that more of it could be amplified under given conditions, so increasing the sensitivity of the assay. It ensured that more of the products were full-length after each cycle, and allowed for shorter synthesis times and lower denaturing temperatures. This meant that the amount of Taq polymerase used in the reaction could be reduced. However, smaller PCR products had a tendency to form heteroduplexes comprising one strand of native cDNA and one strand of control cDNA. These formed in later cycles of the PCR when primers became depleted and product concentration was high, favouring re-annealing of denatured product rather than primer annealing and extension. Heteroduplex formation depleted equal numbers of molecules from each PCR product. A PCR where this occurred was only quantifiable when the PCR products were present in equal amounts. If one product was in excess, the number of molecules removed from each would be disproportionate and the product ratio no longer accurate. As heteroduplexes tended to form in the latter stage of the logarithmic phase, they could be guarded against by reducing the number of cycles to keep the reaction well in this phase. The resultant loss

in sensitivity could be counteracted by ensuring that the specific activity of the primers was high or simply by adding more primers to the reaction.

Precautions Against Contamination of PCRs

A major problem encountered when setting up PCRs was contamination with target DNA. This could be present in the reagents, in the environment, or in the extracted RNA samples as genomic DNA. Amplification of such DNA could lead to falsely elevated levels for target DNA. The degree to which contamination was problematical depended on the sensitivity of PCR required. If the assay was set up to measure picogram amounts of target DNA, the presence of 1 fg of contaminant target would not be detectable. However, if the assay was measuring femtogram levels, contamination with even one or two copies of illegitimate target would have a significant effect on results.

Background contamination was monitored by setting up controls with each batch of PCRs. The presence of genomic DNA in RNA samples was checked by means of 'no-reverse transcriptase' controls. These contained all RNA samples included in the assay and received the same treatment as the regular assay samples except that no reverse transcriptase was added prior to the reverse transcription step. These controls also showed whether any of the reagents used were contaminated with DNA. Contamination of reagents with target RNA could be checked by setting up a 'no-RNA' control, comprising all the constituents of the regular assay sample but with no RNA added. As RNA is so susceptible to enzymatic degradation, this source of contamination was never encountered. Therefore, 'no-RNA' controls were only set up occasionally as an added precaution. A set of assay results were only valid if the negative controls were completely blank after autoradiographic exposure for the same length of time as the regular PCR samples.

To minimize the risk of contamination, RT-PCR was carried out in a specially designated laboratory, away from areas where control cDNAs were handled and PCR products analyzed. DNA samples and reagents, equipment or labcoats from other

laboratories were barred from this room and completed PCRs were never opened there. All necessary stocks of reagents and consumables for PCR were kept in this room.

The enzymes used in the RT-PCR assay were purchased with their reaction buffers and so eliminated the problem of DNA contamination through 'on-site' buffer preparation. For this reason, manufactured kits were used for the final stages of RNA extraction ('RNaid' from BI0 101, Stratech) and for purifying PCR primers after ^{32}P end-labelling ('Mermaid' from BI0 101, Stratech).

No solutions or consumable equipment used in the setting up of RT-PCR assays were autoclaved. Since DNA can withstand autoclave temperatures, this is a potential source of contamination. Bottled sterile water from Gibco was used to prepare other solutions or to dilute reagents. Disposable Eppendorf tubes were found to be essentially DNase- and RNase-free and used directly from the supplier's pack. To prevent RNA or cDNA carry-over between reactions, pre-packed and sterilized, aerosol-resistant pipette tips were used to set up assays. All RT-PCR reagents were aliquoted in small volumes and each aliquot discarded after single use.

Precautions in Handling RNA

If RNA is to be used effectively in any procedure, it is important that it is isolated in an intact and pure form. RNA is degraded at high temperatures and in solutions of high pH. All procedures involving RNA were carried out as quickly as possible and, where possible, on ice. Also, when appropriate, buffers of neutral or slightly acid pH were used.

If any work involving RNA is to be successful, ribonucleases (RNases) must be deactivated. RNases rapidly degrade RNA and are very stable. They are heat-resistant (autoclaving will not destroy them), retain activity over a wide pH range, and can renature after treatment with denaturing agents. RNases are released from disrupted tissues or cells during the extraction procedure, although human skin is also a source.

Gloves were worn for all work involving RNA. The glassware used was purchased new, washed as usual, and then baked at 250^{o}C for 4 hours. All chemicals

used were purchased new and kept separate from other chemicals. They were only handled with gloved hands and, where necessary, with baked spatulas.

All solutions used in the initial stages of RNA extraction were prepared with diethyl pyrocarbonate (DEPC) water and then re-treated with DEPC. DEPC is a non-specific RNase inhibitor, but also reacts with adenine residues in RNA and so has to be removed from treated solutions before they can be used. Solutions were treated by adding 0.2% (v/v) DEPC in a small volume of ethanol as it is not directly miscible in aqueous solution. The solution was shaken vigorously and left at room temperature for at least 10 minutes. DEPC was removed from the solutions by autoclaving, during which it breaks down into CO_2 and ethanol. However, DEPC cannot be used to treat solutions with Tris as it reacts with primary amines. These solutions were prepared using autoclaved DEPC water and then re-autoclaved. Individually-wrapped, sterile disposable pipettes, Pasteur pipettes, syringes, etc. were also used where appropriate. As for RT-PCR procedures, Eppendorf tubes were used directly from the supplier's container, while pipette tips were DEPC treated and autoclaved before use.

The RNA extraction procedure and reactions involving RNA utilized RNase inhibitors. The most successful extraction procedures include deproteinizing agents such as phenol and chloroform, denaturing agents such as SDS and sarcosyl, and chaotropic agents like guanidinium thiocyanate (used in conjunction with β-mercaptoethanol). A specific RNase inhibitor used in the extraction process was vanadyl-ribonucleoside complexes (VRCs, from Pharmacia). These are transition state analogues of the activated 2',3'-cyclic phosphates believed to be formed during catalysis of RNA breakdown by RNases. Unlike other RNA inhibitors, they completely block RNase activity at a concentration of 10 mM. In this study, they were used during DNase treatment of RNA. Placental RNase inhibitor (RNAguard from Pharmacia) was used during *in vitro* transcription reactions for PCR control preparation and in the reverse transcription step of the RT-PCR assay. It is a protein and requires 1 mM DTT to be effective. It forms complexes with RNases and is rendered ineffective by exposure to agents that denature protein.

PCR Primer Choice

The choice of primers used can greatly affect the efficiency of a PCR assay. Ideal primers should meet the following criteria. They should be 18-25 bases long, with a GC content of 50%. Their melting temperatures should be similar and repeats of purines, pyrimidines, or any one base should be avoided. There should be no sequence complementarity between the primers, particularly at their 3' ends, as this could lead to primer dimer formation. Primer dimers compete with the true PCR products for primers and enzyme and so reduce the sensitivity of the assay. Primers with excessive secondary structure (e.g., hairpin loops, etc.) should be avoided as these may anneal to the template poorly.

In this study, the choice of primers had to encompass the following constraints. The p75, BDNF, and L27 control templates were prepared from cDNAs by digesting at a unique restriction enzyme site, filling in the overhangs to form blunt ends, and then re-ligating to give a cDNA 4 bp larger. The *trk*B control template was prepared by partially digesting the cDNA with an enzyme that has two recognition sites so that only the appropriate one was cut and inserting an 8 bp linker into this site. (PCR control template preparation is described in more detail later.) Therefore, the primers chosen had to bind either side of the site containing the additional 4 or 8 bp piece of cDNA. Also, to ensure that amplified control and native templates could be separated by polyacrylamide gel electrophoresis, there was a limitation on the size of the PCR product (the size increase in the PCR control template should be at least 3% of the total length).

For the p75 mRNA assay, the primers were:

(5') 5'-CCTGTGTACTGCTCTATCCTGG-3'

and

(3') 5'-TTGTTCTGCTTGCAGCTGTTCC-3'.

These hybridized 88 bp apart in the chicken p75 sequence (Large *et al.*, 1989) and 92 bp apart in the p75 control template. For the BDNF mRNA assay, the primers were:

(5') 5'-GCAGTCAAGTGCTTTTGGAACC-3'

and

(3') 5'-CATCGACATGTTTGCAGCATCC-3'.

These hybridized 83 bp apart in the pro-sequence sequence of chicken BDNF (Wright and Davies, unpublished data) and 87 bp apart in the PCR control template. For the *trk*B mRNA assay, the primers were:

(5') 5'-AAGGCATCTCCGGTCTACCTG-3'

and

(3') 5'-GGAGTTCAGCGGCAGTTGAAC-3'

These hybridized 98 bp apart in the 3' region of the sequence adjacent to that encoding the tyrosine kinase domain and unique to the full-length *trk*B isoform (Déchant *et al.*, 1993a). For the L27 mRNA assay, the primers were:

(5') 5'-GGCTGTCATCGTGAAGAACATC-3'

and

(3') 5'-CTTCGCTATCTTCTTCTTGCCC-3'.

These hybridized 127 bp apart in the chicken L27 sequence (Lebeau *et al.*, 1991) and 131 bp apart in the PCR control template.

Practical Aspects of the Assay

Initially, *in vivo* levels of p75 mRNA and BDNF mRNA expression were determined in purified chicken sensory, sympathetic and parasympathetic neurons at various stages of embryonic development. In order to compare p75 mRNA or BDNF mRNA levels in these neurons, it was also necessary to measure the level of mRNA encoding the ubiquitous, constitutively expressed ribosomal protein L27. The level of p75 mRNA or BDNF mRNA could then be expressed relative to the level of L27 mRNA. This was found to be a more accurate than relating it to the number of neurons in the cell pellet as determined by haemocytometry. Once p75, BDNF, and L27 mRNA levels had been ascertained in separate RT-PCR assays, it was possible to reverse transcribe controls for all three with the RNA sample. The volumes of the reaction components were adjusted accordingly. A third of the reverse transcription was then

used to assay p75 mRNA, a third to assay BDNF mRNA, and a third to assay L27 mRNA. The different levels of the three mRNAs and the number of PCR products obtained made it impractical to assay all of them in the same PCR. To find amplification conditions that would suit them all and then attempt to separate all the products on a gel would have been impossible. Also, *trk*B and p75 mRNA levels were determined for embryonic chicken vestibular and nodose ganglia, while BDNF mRNA levels were determined for target fields innervated by axons from these ganglia. Differences in the size and number of these ganglia and tissues were taken into account by expressing the mRNA levels relative to those for L27 mRNA.

In vitro regulation of p75 mRNA expression was then determined using neuronal cultures. These experiments were carried out using cultures of embryonic chicken sympathetic chain and DRG neurons and took two forms. Firstly, that of a dose response in which cultures were set up containing different concentrations of a trophic factor. Secondly, cultures were set up containing, for example, a particular trophic factor or combination of factors at fixed concentrations. At least two cultures were set up for each condition. The number of neurons in each culture was counted at either 36 or 48 hours, the neuronal RNA then extracted, and the p75 mRNA levels assayed. p75 mRNA levels were expressed as fg/neuron.

The effect of BDNF on *in vitro* regulation of *trk*B mRNA was studied in stage 19 chicken nodose neurons. Cultures were set up in duplicate with and without added BDNF. The number of neurons in two dishes with and two dishes without BDNF was counted at 24, 36, 48, 60, and 72 hours, the neuronal RNA extracted, and *trk*B mRNA levels determined. *trk*B mRNA levels were expressed as fg/neuron.

In order to determine how much control template to use, a fixed amount of RNA sample was assayed with different amounts of control template. After autoradiographic exposure, the reaction where the signals of both control and sample products were of equal or near equal intensity was taken as being the most accurate. This reflected the fact that there were similar amounts of each species in the reaction and so reduced amplification bias of one of them. It also meant that the autoradiographic

exposure time required to visualize each signal was similar, making it easier to ensure that the response of the X-ray film remained in its linear range. The ratio of the autoradiographic signals could then be determined and the level of target mRNA in the RNA sample calculated.

Routinely, 1 µl of each RNA sample was used. Once PCR control levels had been determined, batches of samples were assayed such that the same amount of control template could be added to each RT-PCR. In some cases, RNA samples had to be diluted or more than 1 µl added to allow all the RNA samples from one experiment to be assayed using the same amount of cRNA control.

Preparation of RT-PCR Control Templates

The chicken p75 cDNA, from which the p75 PCR control was prepared, was a gift from Thomas Large (Department of Physiology and the Howard Hughes Medical Institute, University of California). It comprised 1.8 kb of chicken p75 cDNA subcloned into the EcoR 1 site of the pGem-3Z vector. The PCR control was constructed by, firstly, digesting the plasmid at a unique Eag 1 restriction site located in the p75 insert, generating cohesive ends with 5' overhangs 4 bases in length. The overhangs were then filled in using the Klenow fragment of DNA polymerase 1 and the resultant blunt ends religated (Maniatis *et al*, 1989). This inserted 4 bp into the sequence. In order to produce a smaller template for *in vitro* transcription, the p75 cDNA was then cleaved at Ava 1 sites on either side of the filled in Eag 1 site. This produced a fragment of cDNA approximately 700 bp in length. The Ava 1 sites of the fragment were filled in and the fragment blunt-end ligated into the filled in Ava1 sites of the pGem-3Z vector (the cohesive ends of insert and vector were not compatible after Ava 1 cleavage). The fragment of p75 cDNA subcloned corresponds to bases 483-1,177 of the published sequence (Large *et al.*, 1989). The p75 cDNA insert was in the sense orientation with respect to the SP6 RNA polymerase promoter site of the vector.

The chicken BDNF control template was prepared from cDNA subcloned into the EcoR 1 site of the pGem-4Z vector, in the sense orientation with respect to the

vector SP6 RNA polymerase promoter site. The PCR control was constructed by cleaving the cDNA at a unique Xho 1 site, filling in the 5' overhangs and re-ligating the resultant blunt ends.

463 bp of full-length *trk*B cDNA were obtained by reverse transcription of total RNA from E11 chicken DRG and subsequent PCR amplification using primers based on the published chicken *trk*B sequence (Déchant *et al.*, 1993a). The primers were:

(5') 5'-TAGGAATTCGTACAGCACAGACTACTACAGG-3'

and

(3') 5'-CAAGGATCCCATTAATACTGTCAAAGTTAAG-3'

They were designed with restriction sites (shown underlined) at their 5' ends to facilitate subcloning and with 3 bp of the *trk*B sequence prior to these to prevent the ends of the DNA from 'breathing' during digestion with these enzymes. The 5' primer bore an EcoR 1 site and the 3' primer bore a BamH 1 site. These primers isolated a fragment of cDNA corresponding to bases 2,214 to 2,677 of the published sequence, spanning parts of the tyrosine kinase domain and 3' untranslated region. The *trk*B cDNA was subcloned into the EcoR 1 and BamH 1 sites of the pGem-4Z vector. The *trk*B cDNA insert was in the sense orientation with respect to the pGem-4Z SP6 RNA polymerase promoter site. The first stage in the preparation of the PCR control construct was the partial digestion of the *trk*B/pGem-4Z DNA using Xmn 1 which cleaves to produce blunt ends. The *trk*B/pGem-4Z construct contained two Xmn 1 sites: one in the ampicillin resistance gene of the pGem-4Z vector and one in the 3' untranslated region of the *trk*B insert. The construct was modified at the latter restriction site to produce the PCR control template using the following procedure. Partial digestion gave a mixture of linearized *trk*B/pGem-4Z fragments that had been cut at either one Xmn 1 site or the other. The digest was then run on an agarose/TAE gel to isolate the partially digested DNA fraction from any DNA that had been cleaved at both Xmn 1 sites. The bands of partially digested DNA were then excised from the gel, purified using the Geneclean kit, and CIAP treated as described previously. 8 bp phosphorylated Cla 1 linkers (Biolabs) were blunt-end ligated into the cleaved Xmn 1

sites of the partially digested DNA. The ligation mixture was used to transform competent XL1 Blue (*E. coli*) bacterial cells. The cells were plated on L agar with ampicillin. This eliminated all cells bearing constructs with a Cla 1 linker inserted in the ampicillin resistence gene of the pGem-4Z vector. A construct with a Cla 1 linker in the *trk*B fragment was then identified and used to prepare the PCR cRNA control template.

359 bp of L27 cDNA were obtained by reverse transcription of total RNA from E11 chicken DRG and subsequent PCR amplification using primers based on the published L27 cDNA sequence (Lebeau *et al.*, 1991). The primers were:

(5') 5'-AAGGTCGACAAGGTGGTGCTGGTGCTC-3'

and

(3') 5'-AAGGGATCCTTATTCTTGCCAGTGTTG-3'.

As for the *trk*B primers, they were designed with restriction sites (shown underlined) at their 5' ends for subcloning purposes and with 3 bp of the L27 sequence prior to these to assist enzymatic digestion. The 5' primer bore a Sal 1 site and the 3' primer bore a BamH 1 site. These primers isolated a fragment of cDNA corresponding to bases 51 to 410 of the published sequence. The L27 cDNA was subcloned into the Sal 1 and BamH 1 sites of the pGem-3Z vector. The PCR control construct was prepared by cleaving the L27 cDNA at a unique Sty 1 restriction site, filling in the 5' overhangs, and re-ligating the blunt ends. The L27 cDNA insert was in the sense orientation with respect to the pGem-3Z SP6 RNA polymerase promoter site.

Synthesis of Control cRNA

All experiments within this study used the same batch of PCR cRNA control templates that were transcribed, purified, and quantified in one procedure. It was hoped that this would provide some degree of uniformity with respect to the controls.

The cDNA constructs prepared were first linearized with a suitable restriction enzyme such that 'run-off' transcripts could be synthesized using SP6 RNA polymerase. The enzyme chosen must produce either cohesive termini with 5' overhangs or blunt-ended termini as cohesive termini with 3' overhangs tend to give

erroneous transcripts. The linearized template was phenol-chloroform extracted twice and ethanol precipitated. After pelleting, the DNA was washed in 70% ethanol and resuspended in DEPC-treated water so that the DNA concentration was approximately 1 μg/μl.

Control RNA transcripts were synthesized in a 200 μl reaction comprising: 40 μl 5x transcription buffer (supplied with SP6 RNA polymerase from Gibco BRL), 8 μl each of 10 mM ATP, 10 mM CTP, 10 mM GTP, and 10 mM UTP, 8 μl of 100 mM DTT, 5 μl of RNAguard (40 units/μl), 5 μl of linearized DNA template (5 μg), 107 μl of DEPC-treated water, and 3 μl of SP6 RNA polymerase (16 units/μl). Following incubation at 37°C for one hour, a further 3 μl of SP6 RNA polymerase were added and the reaction allowed to continue at 37°C for another hour.

The reaction was then made 10 mM with respect to VRCs and 50 units of FPLC-pure RNase-free DNase 1 (Pharmacia) were added to digest the DNA template. The following purification steps were carried out in the PCR room. The DNase 1 reaction was stopped by the addition of 600 μl of high salt binding solution (3 M $NaClO_3$, supplied with the BIO 101 RNaid kit from Stratech). 50 μl of RNaid matrix were then added, mixed thoroughly, and this left at room temperature for 10 minutes to allow the RNA to bind to the matrix. The matrix with bound RNA was sedimented by brief centrifugation at 13,000 rpm and the supernatant discarded. It was then washed twice with 1 ml of ice-cold RNaid wash solution. The RNA was eluted from the matrix by adding 500 μl of sterile water (Gibco BRL) and incubating at 60°C for 5 minutes. Following brief centrifugation to sediment the matrix, the RNA solution was removed to a fresh Eppendorf tube.

A small amount of the RNA solution was used to determine the RNA concentration spectrophotometrically (for RNA, an OD_{260} of 1 was taken as 40 μg/ml). This synthesis procedure usually produced 200-300 μg/ml of cRNA. The remainder of the cRNA was diluted and precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 5.2), 3 volumes of absolute ethanol, and *E. coli* tRNA, such that the final ethanolic mixture was 1 ng/μl with respect to the transcript RNA and 100 ng/μl with

respect to *E. coli* tRNA. 50 µl aliquots (50 ng of transcripts) of the ethanol-precipitated RNA were then stored at -20°C until required.

Extraction of Total RNA for PCR

This method is based on the acid guanidinium thiocyanate-phenol-chloroform extraction procedure (Chomczynski and Sacchi, 1987) and has been adapted for use with PCR. It is suitable for extraction of total RNA from cells on a 35 mM tissue culture dish or from 10-50 ganglia.

The cells were first lysed by the addition of 500 µl of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate at pH 7.0, 0.5% L-laurylsarcosine, and 0.1 M β-mercaptoethanol). For neuronal cultures, once the F14 culture medium had been removed, solution D was applied directly to the culture surface, swirled aroumd the culture dish for 30 seconds, and transferred to an Eppendorf tube. For purified neurons and ganglia, solution D was added to the Eppendorf tube in which they had been stored frozen. The lysates were placed on ice and the following ice-cold solutions added to them in the order given: 1 µl of 1 mg/ml *E. coli* tRNA; 50 µl of 2 M sodium acetate, pH 4.0; 500 µl of water-saturated phenol; and 120 µl of 24:1 chloroform/isoamyl alcohol. After each of the first three additions, the tube contents were mixed by inversion; after the final addition, they were mixed by vigorous shaking for 10 seconds. The mixture was then cooled on ice for 15 minutes and centrifuged at 14,000 rpm for 15 minutes at 4°C. The upper aqueous phase was removed to a fresh Eppendorf and RNA was precipitated by the addition of 2 volumes of absolute ethanol. Precipitation was allowed to take place at -20°C for at least one hour but usually overnight.

The precipitated RNA was pelleted by centrifuging at 14,000 rpm for 30 minutes, washed thoroughly with 70% ethanol, and air dried (vacuum drying tends to over-dry the pellets, making the RNA difficult to resuspend). The RNA pellet was resuspended in 20-50 µl of a solution containing 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 50 mM VRCs, and then incubated with 20-30 units of RNase-free DNase 1

(Pharmacia) at 37°C for 1-2 hours.

The following procedures were then carried out in the PCR room and entailed a further purification of the RNA using the BIO 101 RNAid kit (Stratech). Three volumes of high salt binding solution (3 M $NaClO_3$, from the kit) were added to the RNA and VRC mixture, followed by 5-25 µl of the RNaid binding matrix. The actual volume of binding matrix added is dependent on the amount of cells from which the RNA is extracted, e.g., 5 µl of matrix for approximately 1,000 E7 chicken sympathetic neurons. After thorough mixing, the RNA and matrix were left at room temperature for 5-10 minutes to allow for the binding of the RNA to the matrix. The mixture was centrifuged briefly at 13,000 rpm to sediment the matrix with bound RNA. The pellet was washed twice with 500 µl and then 750 µl of ice-cold RNaid wash solution. After a final centrifugation at 13,000 rpm, as much of the wash solution as possible was removed and the matrix resuspended in 10-50 µl of sterile distilled water (Gibco BRL). The RNA was eluted from the resuspended matrix by incubating at 60°C for 5 minutes. The matrix was pelleted by centrifugation at 13,000 rpm for one minute, the RNA solution was removed to a fresh Eppendorf tube and stored at -70°C.

Preparation of Labelled Primers

The primers were labelled at their 5' ends in a 40 µl reaction containing 4 µl 10x T4 polynucleotide kinase buffer (0.5 M Tris at pH 7.6, 100 mM $MgCl_2$, 1 mM spermidine, 50 mM DTT, 1 mM EDTA at pH 8.0), approximately 2 µg of each primer, 30 µl of ^{32}P γATP (3,000 Ci/mmol, 10 mCi/ml), and 4.5 µl of NBL T4 polynucleotide kinase (10 units/µl). The reaction was mixed thoroughly and incubated at 37°C for 45 minutes, after which a further 5 µl of T4 polynucleotide kinase were added, followed by a second 45-minute incubation at 37°C.

The labelled primers were recovered from the rection mixture using the BIO 101 Mermaid oligonucleotide purfication kit (Stratech). Three volumes of Mermaid high salt binding solution were added to the reaction followed by 50 µl of Mermaid glass matrix. This was mixed thoroughly and left for 15 minutes at room temperature to permit the

primers to bind to the glass matrix. The mixture was centrifuged briefly at 13,000 rpm to sediment the glass matrix plus bound primers. The pellet was washed twice with 500 µl of the Mermaid wash solution, and, after removal of as much of the wash solution as possible, the primers were resuspended in 250 µl of sterile distilled water (Gibco BRL). They were eluted from the glass matrix by incubation at 60°C for 10 minutes. The glass matrix was sedimented by centrifugation at 13,000 rpm, and the primer solution transferred to a fresh Eppendorf tube and stored at -20°C until required.

Reverse Transcription

Total RNA was reverse transcribed in a 10 µl reaction using Gibco BRL Superscript reverse transcriptase in the supplied buffer plus 0.5 mM dNTPs (Ultrapure, from Pharmacia), 10 mM DTT (also supplied with the enzyme), 10 M random hexanucleotides (Pharmacia), 10 units of RNAguard (Pharmacia), and the appropriate amount of control template. The reaction was incubated for 45 minutes at 37°C.

A 'master mix' was prepared of the components that were common to both regular and negative control reactions. Using the same mix for one batch of experiments provided some degree of uniformity. The volume of 'master mix' used in each reaction was 8.5 µl, and so the total volume (v) for n number of reactions was

$$v\ (\mu l) = (n+1) \times 8.5$$

The 'master mix' was prepared for (n+1) reactions to allow for inaccuracies in pipetting when aliquoting it into individual reaction tubes.

The 'master mix' was prepared as follows:

2 x (n+1) µl 5x supplied reaction buffer

1 x (n+1) µl 0.1M DTT

1 x (n+1) µl 5mM dNTPs

1 x (n+1) µl 100M random hexanucleotides

0.25 x (n+1) µl RNAguard

+ appropriate concentration of cRNA control in (n+1) µl

+ sterile distilled water (Gibco BRL) to volume v

8.5 µl of the 'master mix' was dispensed into individual 0.5 ml Eppendorf tubes and the RNA samples (usually 1 µl) were then added. In cases where one RNA sample was being amplified with different amounts of control template, the total RNA was added to the 'master mix' prior to its being dispensed and the control template dilutions added to the individual tubes. If desired, 'no RNA' controls could be set up by preparing the 'master mix' with all its components except the cRNA control, dispensing 7.5 µl, and making up the volume to 9.5 µl with 2 µl of sterile distilled water (Gibco BRL). The appropriate amount of cRNA control could then be added to the remainder of the 'master mix'. The tubes were placed in a water bath at 37°C and equilibrated for one minute. 0.5 µl (100 units) of Superscript reverse transcriptase (Gibco BRL) was added and the reactants mixed in the pipette tip. Superscript was not added to the 'no-reverse transcriptase' control reactions. The tubes were centrifuged briefly, returned to the 37°C water bath, and incubated for 45 minutes.

When reverse transcribing more than one cRNA control in the same reaction, the reactant volumes and amount of total RNA added were adjusted accordingly, e.g., for the reverse transcription of p75, BDNF, and L27 cRNA controls in the same reaction, the total reactant volume would be 30 µl and 3 µl of total RNA would be added.

PCR

After reverse transcription, the reactions were centrifuged briefly to return the

contents to the bottom of the tube. The RNA template was then destroyed by heating the reactions at 98°C for 5 minutes in the heating block of a thermal cycler. The reactions were then quenched on ice and briefly centrifuged once more.

The components for PCR were then added to the reverse transcriptions. These were prepared as a 'master mix'. The additional volume of PCR mix added to each reverse transcription was 40 µl and so the total volume (v) of the 'master mix' for n number of PCRs was (n+1) x 40 µl. The composition of the 'master mix' was as follows and was adequate for the amplification of 0.5 fg-10 pg of initial target cDNA:

4 x (n+1) µl 10x supplied NH_4 reaction buffer

1 x (n+1) µl 5 mM dNTPs (Ultrapure, from Pharmacia)

X x (n)+1) µl labelled primer mix

Y x (n+1) µl 50 mM $MgCl_2$ (supplied with Bioline Taq polymerase)

0.5 x (n+1) µl Biotaq (5 units/µl, from Bioline)

+ sterile distilled water to volume v

The 10x NH_4 PCR buffer contained no $MgCl_2$. Volumes 'X' and 'Y' were specific for each target template and needed to be optimized.

Some variation was found in the efficiency to which individual primer pairs labelled with ^{32}P. An insufficient volume of labelled primers decreased the sensitivity of the assay, while a great excess of primers generated background signals from mispriming. It was, therefore, necessary to find the optimum volume required for each pair. This was as follows: for the p75 and *trk*B mRNA assays, 6 µl of purified labelled primers were used, while for the BDNF and L27 mRNA assays, 5 µl were used.

The concentration of free Mg^{2+} ions in the PCR was found to influence the efficiency and fidelity of the Taq polymerase. Since dNTPs and primers bind Mg^{2+} and the extent of binding is dependent on the concentration of these species, the concentration of $MgCl_2$ required in the PCR had to be optimized for each template and primer pair. The concentrations of additional $MgCl_2$ required by each assay were as

follows: for the p75 mRNA assay, 1.85 mM $MgCl_2$ were added, for the BDNF mRNA assay, 1 mM was added, and for the *trk*B and L27 mRNA assays, 1.5 mM were added.

After 40 µl of the PCR 'master mix' were added to each reverse transcription, the two solutions were mixed and overlaid with 40 µl of mineral oil. The tubes were then placed in a thermal cycler for amplification by rounds of denaturing, primer annealing, and DNA synthesis. Where, for example, p75, BDNF, and L27 cRNA controls were reverse transcribed together in a 30 µl reaction, 10 µl aliquots were dispensed for each PCR assay.

The optimum conditions for amplification were determined for each assay and were found to be template and primer specific, and also influenced by the initial amount of target cDNA. In general, the amplification conditions utilized a few 'high stringency' cycles followed a larger number of 'low stringency' cycles. The 'high stringency' cycles comprised a high-temperature denaturing step to facilitate the separation of larger cDNA species in the initial stages of amplification. They also comprised a high-temperature annealing step with a short annealing time to reduce non-specific amplification through mispriming and a short synthesis step to encourage the synthesis of a small PCR product. The 'low stringency' cycles were designed for rapid amplification once a good level of target cDNA had been synthesized by the high stringency parameters. These comprised a lower denaturing temperature to favour the strand separation of a short PCR product, a lower annealing temperature with a longer annealing time to improve primer annealing, and a longer synthesis time for greater product yields.

The number of amplification cycles used depended on the initial amount of target cDNA; the lower the concentration of target template, the more cycles were necessary. Whatever the number of cycles used, it was important to ensure that the PCR remained in the logarithmic phase and did not plateau so that the ratio between target and control templates was accurate.

Chicken p75 target cDNA was amplified by 8 cycles of 94°C for 60 seconds,

54°C for 90 seconds, and 72°C for 45 seconds followed by 13-19 cycles of 91°C for 60 seconds, 52°C for 120 seconds, and 72°C for 120 seconds. There was a final 72°C incubation for 10 minutes. These conditions were optimal for the amplification of 2-100 fg of both target and control templates.

Chicken BDNF target cDNA was amplified by 8 cycles of 94°C for 60 seconds, 54°C for 60 seconds, and 72°C for 60 seconds followed by 12-17 cycles of 91°C for 60 seconds, 52°C for 75 seconds, and 72°C for 90 seconds. There was a final 72°C incubation for 10 minutes. These conditions were optimal for the amplification of 0.5-5 fg of both target and control templates.

Chicken *trk*B target cDNA was amplified by 8 cycles of 94°C for 60 seconds, 56°C for 45 seconds, and 72°C for 45 seconds followed by 14-21 cycles of 91°C for 60 seconds, 54°C for 60 seconds, and 72°C for 60 seconds. There was a final 72°C incubation for 10 minutes. These conditions were optimal for the amplification of 0.5-10 fg of both target and control templates.

Chicken L27 target cDNA was amplified by 8 cycles of 94°C for 60 seconds, 60°C for 45 seconds, and 72°C for 45 seconds followed by 11-15 cycles of 91°C for 60 seconds, 58°C for 90 seconds, and 72°C for 180 seconds. There was a final 10-minute incubation at 72°C. These conditions were optimal for the amplification of 500 fg-10 pg of both target and control templates.

Electrophoresis of PCR Products

The RT-PCR products were separated by electrophoresis on non-denaturing polyacrylamide gels. The minimum concentration of polyacrylamide found to completely separate two species with a size difference of around 4% was 7% of 19:1 acrylamide/bis.

The Gibco BRL V-15.17 vertical gel apparatus was used. The gel dimensions were 20 x 25 cm with a thickness of 0.8 mm. The comb chosen could hold 20 10 µl samples. The gel plates were thoroughly cleaned and one siliconized. The apparatus was then assembled according to the manufacturer's instructions.

The gel solution, comprising 7% acrylamide/bis and 1x TBE, was prepared in a total volume of 250 ml and stored at 4°C for up to one month. 30 ml of gel solution were first de-gassed under vacuum. 60 µl of freshly-prepared 20% ammonium persulphate and 45 µl of TEMED were then added and the solution mixed and introduced between the glass plates of the assembled gel apparatus using a 25 ml disposable syringe. The gel was then left to polymerize for 30 minutes at room temperature.

After polymerization, the comb was carefully removed from the gel and the wells flushed free of unpolymerized acrylamide using 1x TBE in a 25 ml syringe with a 19 gauge needle. The gel was placed in the apparatus, the buffer reservoirs filled with 1x TBE, and the wells flushed again to remove air bubbles.

Prior to loading, 10 µl of 6x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol) were added to the 50 µl reactions. For the p75, *trk*B, and L27 mRNA assays, 10 µl/PCR were loaded onto the gel. The smaller PCR products of the BDNF mRNA assay tended to run more diffusely and so 5 µl/PCR were loaded to give 'tighter' bands.

After running the products as near to the bottom of the gel as possible to give maximum resolution, the gel was removed from the apparatus and the plates carefully separated so that the gel remained attached to the non-siliconized plate. The gel was then taken up onto Whatman 3MM paper, covered with Saran Wrap, and dried down on a slab gel drier for 30 minutes at 60°C.

The dried gel was then autoradiographed. The relative intensities of the autoradiographic signals for control cRNA and mRNA products were then determined using a Molecular Dynamics laser densitometer with ImageQuant software.

Dissection and Cell Culture Techniques

Dissection Techniques

Embryos were obtained from Fertile White Leghorn chicken eggs incubated at 38°C in a forced-draft incubator. For the studies using purified neurons, the sensory

ganglia were dissected after 10, 12, and 14 days of incubation; the sympathetic chain ganglia after 10, 12, 14, and 16 days; and the parasympathetic ciliary ganglia after 10, 12, and 16 days. For the studies using cultured neurons, sympathetic chain ganglia were dissected after 7 and 14 days of incubation, dorsal root ganglia (DRG) after 14 days, and dorsomedial trigeminal ganglia (DMTG) and trigeminal mesencephalic nuclei (TMN) after 10 days. For the study of *trk*B and BDNF mRNA expression, nodose and vestibular ganglia, heart, otic vesicle, and hindbrain were dissected from staged (Hamburger and Hamilton, 1951) chicken embryos early in development. Further details of the tissue dissection and cell culture techniques involved in this work are given in Chapter 5.

A detailed description of tissue dissecton is given in Davies (1988b) but a brief outline is shown below. All procedures were carried out using standard sterile technique in a laminar flow hood. Embryos were removed from the eggs by first holding the egg with the blunt end uppermost (where the airspace is located). The shell was wiped with 70% ethanol and allowed to dry, then cracked in a line around the airspace with forceps and the portion of shell removed along with the membrane lining the airspace. Embryos were removed using curved forceps placed beneath the neck, decapitated, and collected in a large petri dish.

The dissections were carried out in HEPES-buffered (20 mM) Hanks' balanced salt solution (Hanks, from Gibco BRL) under a stereomicroscope at x20 magnification. A fibre optic light source was used to avoid overheating the specimens. Electrolytically-sharpened tungsten needles were used to complete the dissections.

Sympathetic Ganglia and Dorsal Root Ganglia (DRG)

Abdominal and thoracic viscera were removed using watchmaker's forceps and the posterior abdominal and thoracic walls washed with Hanks from a Pasteur pipette. Blunt forceps were used to slit the connective tissue sheath lying around the spinal column and the sheath carefully torn back. The sympathetic chain was carefully teased away from the spine using forceps, taking care not to separate the chain from the

underlying DRG. The chain was removed from mid-thoracic to sacral regions and separated from the connective tissue using sharpened tungsten needles. DRG (shown in figure 1, part A) were removed from the lumbosacral region by passing the pointed ends of forceps between the ganglia and spinal cord, so cutting the spinal roots. Each ganglion was removed using watchmaker's forceps, holding the nerve distal to the ganglion. The ganglia were collected in a petri dish of Hanks and associated nerves removed using tungsten needles. Between 20-30 of each ganglia were collected.

Cranial Sensory Ganglia

The locations of these ganglia in the chicken are shown in figure 1, part B.

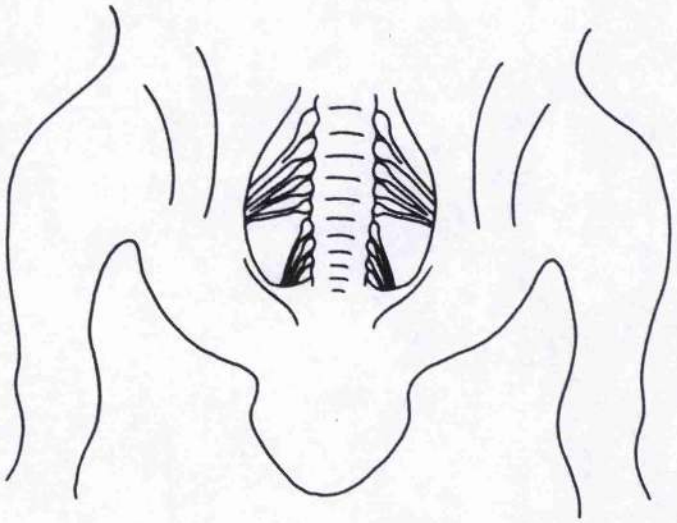
Nodose ganglia are located at the base of the neck, on either side of midline tissues in front of the vertebral column. They are glistening white and spindle-shaped in appearance with the attached vagus nerve passing rostrally. The ganglia were removed by taking hold of the vagus nerve with watchmaker's forceps. Associated nerve fibres were removed with tungsten needles.

The jugular, trigeminal, and vestibulo-acoustic (vestibular) ganglia were dissected from the cranial base after removal of the brain. A no. 15 scalpel blade was used to dissect the cranial base as shown in Davies (1988b). Tungsten needles were then used to complete the dissection of ganglia from the resultant tissue blocks, each ganglion being identified by its characteristic shape (see figure 1, part B). The trigeminal ganglion was then subdissected using tungsten needles into the regions containing dorsomedial neurons and ventrolateral neurons as described in Davies (1988b). 20-30 ganglia of each type were dissected out.

Trigeminal Mesencephalic Nucleus (TMN)

This was dissected from the midbrain. The cranial vault was removed with watchmaker's forceps and the brain removed by passing a small spatula between it and the cranial base. 20-40 brains were collected in Hanks and these were transferred to a fresh petri dish of Hanks. The dissection was completed using tungsten needles as

A



B

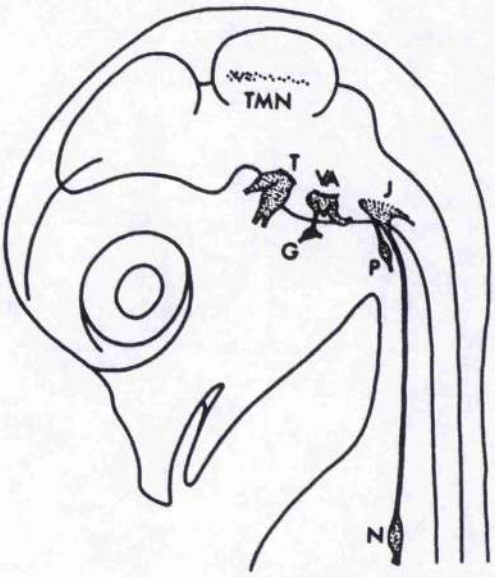


Figure 1 (A) Camera lucida drawing of the ventral aspect of the lumbosacral region of an E10 chick embryo after evisceration showing the location of the dorsal root ganglia. (From Davies, 1988b.) (B) Schematic illustration of an E10 chick embryo showing the locations of the cranial sensory ganglia. TMN, trigeminal mesencephalic nucleus; T, trigeminal ganglion; G, geniculate ganglion; VA, vestibulo-acoustic (vestibular) ganglion; J, jugular ganglion; P, petrosal ganglion. (From Davies and Lindsay, 1985.)

described in Davies (1988b). Briefly, the midbrain was isolated by two coronal incisions and the pia mater peeled off, initially from the ventral aspect. The TMN is located in the cerebral aqueduct. The roof of the cerebral aqueduct was then dissected from the midbrain and the median part of the TMN subdissected.

Ciliary Ganglia

The parasympathetic neurons of the ciliary ganglion were removed from embryos by simply scoring around the edge of the eye-ball with blunt forceps and then removing the eye from the socket. Care was taken to not damage the eye and the underlying tissues in the eye socket. The ciliary ganglia, situated near the stalk of the optic nerve, were then removed from the posterior part of the eye using blunt forceps. The ganglia were then cleaned from connective tissue using tungsten needles.

Tissue Dissociation

Ganglia used to prepare samples of purified neurons and for cell culture were first dissociated into a single cell suspension. The dissected ganglia were transferred to a 10 ml conical tube containing 1-2 ml of calcium- and magnesium-free, phosphate-buffered isotonic saline (CMF-PBS, from Gibco BRL). This was agitated briefly to wash the tissue and the CMF-PBS removed with a pipette. 1.8 ml of fresh CMF-PBS were added plus 200 µl of 1% trypsin (Worthington) in CMF-PBS (stored in 200 µl aliquots at -20^{o}C).The tube was partially immersed in a water bath at 37^{o}C for 15-20 minutes and agitated at intervals. The exact time of incubation needed to be optimized for different batches of trypsin or ages of ganglia. This was to minimize neuronal damage caused by over-trypsinization or by the vigorous trituration required to dissociate under-trypsinized tissue.

After trypsinization, most of the liquid was removed with a pipette and the tissue washed with 2 x 10 ml of Hanks plus 10% heat-inactivated horse serum (HIHS, Gibco BRL) to remove and inactivate any remaining trypsin. This was followed by washing in 5 ml of CMF-PBS. Centrifugation at 2,000 x g for 1-2 minutes facilitated

removal of the liquid between washes.

The tissue was then dissociated into a single cell suspension by trituration. This was carried out using a siliconized Pasteur pipette, the tip of which had been heated in a bunsen burner to produce a fine bore. The tissue was drawn up into the pipette in about 1 ml of CMF-PBS, The tip of the pipette was then placed near the bottom of the tube and the contents of the pipette slowly expelled with firm pressure. This was repeated two or three times until the tissue was fully dissociated. The progress of dissociation was checked by examining a drop of the dissociate on a slide under an inverted phase microscope.

Separation of Neurons from Non-Neuronal Cells

Where possible, it was important to purify neuronal cells from other cells found in the ganglia. These non-neuronal cells may not only synthesize and release neurotrophic factors in culture, but may also express the mRNAs being quantified in neurons.

Neurons were purified free from non-neuronal cells by differential sedimentation (Davies, 1988b). This made use of the fact that larger neuronal cells sediment more quickly in liquid medium than smaller non-neuronal cells. It is, therefore, ineffective at early stages of development when neuronal and non-neuronal cells are similar in size.

The sedimentation procedure was carried out in a 100 ml cylindrical glass dropping funnel with a ground glass outlet tap. Prior to use, the funnel and tap were autoclaved separately with pieces of aluminium foil on the top, spout, and tap regions. The funnel apparatus was assembled and filled to a height of about 10 cm with Ham's F14 plus 10% HIHS. It was then clamped vertically in a stand and this left on a vibration-free surface at 2±0.5^{o}C overnight. The Ham's F14 was purchased from Gibco BRL as a powder and prepared using sterile, bottled water also from Gibco BRL. For 500 ml, 1 g of $NaHCO_3$ (F14 is CO_2 buffered), 30 mg of penicillin, and 50 mg of streptomycin were added, and the pH adjusted to 7.4. The medium was sterilized

through a 0.22 μm filter and stored at 4°C for up to one month. After addition of HIHS, the medium was re-filtered.

The dissociated cell suspension was made up to a volume of about 2 ml in CMF-PBS. The dropping funnel was removed from the cool incubator and the cell suspension carefully layered by running down the inside of the funnel. The foil was replaced on the top of the funnel and this returned to the cool incubator for a further hour. The funnel was then carefully taken out of the incubator, the foil removed from the spout, and 5 ml aliquots run off into sterile tubes. To determine which fractions contained only neurons, 500 μl samples of these aliquots were placed in a 24-well plate (16 mm wells) and examined under a phase contrast microscope.

For studies using purified neurons not placed in culture, the neuronal fractions were pooled and aliquots containing approximately equal numbers of neurons were centrifuged at 2,000 x g for 2 minutes and the supernatant removed prior to rapid freezing in liquid nitrogen. The neurons were stored at -70°C until required. For the studies using purified, cultured neurons, the neuronal fractions were again pooled and the neurons placed in culture as follows.

Cell Culture

For most of these studies, neuronal cultures were set up in 35 mm diameter plastic tissue culture dishes from Nunc (other details are given as appropriate). Neurons do not attach well to tissue culture plastic itself, which means that they are unable to extend neurites and survive in culture. It was, therefore, necessary to coat the dishes with laminin, an extracellular matrix protein, before placing the neurons in culture. The dishes were first coated with poly-DL-ornithine (P-ORN) which facilitates the adsorption of the laminin.

A 0.5 mg/ml P-ORN solution was prepared by dissolving powdered P-ORN (Gibco BRL) in 0.15 M borate buffer (0.15 M boric acid adjusted to pH 8.4 with NaOH). The P-ORN was sterilized by passing through a 0.22 μm filter and was stored at 4°C for up to one week. To coat the dishes, 1.2 ml of P-ORN were added to each

dish and this left overnight at room temperature. The P-ORN was removed using a Pasteur pipette attached to a suction line and the dishes washed three times with sterile water. 1-2 ml of sterile water were added to each dish, swirled around, and removed with the suction line. The dishes were left in the laminar flow hood to air dry for about 15 minutes. The lids were then replaced and the dishes could be used immediately or stored for several days if desired.

1 mg/ml laminin (Gibco BRL) was stored in 20 µl aliquots at -70°C. The laminin was first thawed at 4°C and diluted to 20 µg/ml with sterile F14 without serum or CMF-PBS. 150 µl of diluted laminin were placed in the centre of each P-ORN-coated dish and spread across most of the base with a sterile pipette tip. The dishes were then placed in a humidified incubator at 37°C for a minimum of 4 hours or overnight.

It was necessary to wash excess laminin off the dishes immediately before use with 2 x 2 ml F12 medium plus 10% HIHS. As for F14, F12 was purchased in powdered form from Gibco BRL and was made up using autoclaved double-distilled water. For one litre, 100 mg of streptomycin and 60 mg of penicillin were added and the pH adjusted to 7.4. The medium was then sterilized by passing through a 0.22 µm filter. After the addition of 2 ml F12 to each dish, the medium was swirled and removed using the suction line. As it was important that the dishes did not dry out in between washes, F12 was removed from only 4 dishes at a time and the second 2 ml of medium added. After the second wash, the F12 plus 10% HIHS was replaced by 1 ml of F14 plus 10% HIHS, measured accurately using a micropipette.

The appropriate growth factors were added to at least duplicate dishes for each treatment and/or time point at double the required final concentration. For dose response experiments, the desired trophic factor dilutions were prepared in culture medium at double the required concentration. Medium was removed from the washed dishes and 1 ml of each dilution added to at least duplicate dishes. In both cases, the dissociated neurons were placed in a suitable volume of F14 plus 10% HIHS in a screw-top tube, evenly distributed in the medium by a gentle movement, and added to

each dish in two 500 µl aliquots using a micropipette. The neurons were usually plated at a density of 800-1,000 neurons/dish. The cells were evenly distributed in the dishes by a gentle movement backwards and forwards and from side to side. The cultures were then incubated for the desired times at 37°C in 3.5% CO_2 in a humidified incubator.

The neurons were quantified as follows. Each dish was centrally positioned over a 12 x 12 mm graticule comprising 2 mm squares and the number of neurons located in the grid counted under an inverted phase microscope at x200 magnification. From this, the number of neurons in each dish as a whole could be estimated for mRNA quantitation by RT-PCR. For neuronal survival studies, the number of neurons in the grid that were attached to the P-ORN/laminin substratum was first determined for a few dishes at 6 to12 hours after plating. The number of surviving, process-bearing neurons was then determined for all the dishes at 48 hours after plating. This was expressed as a percentage of the initial 6 to12-hour count.

CHAPTER 3

In Vivo p75 Expression in Sensory, Sympathetic, and Parasympathetic Neurons and *In Vitro* Regulation of p75 Expression in Sympathetic and Dorsal Root Ganglion Neurons

Introduction

At the time of this study, the role of the common low-affinity p75 receptor in mediating the effects of neurotrophic factors was unclear and the available evidence conflicting. It had been shown that p75-deficient NIH 3T3 fibroblasts transfected with each of the *trk* receptors can survive (Glass *et al.*, 1991) and proliferate (Cordon-Cardo *et al.*, 1991; Lamballe *et al.*, 1991; Glass *et al.*, 1991; Kaplan *et al.*, 1991b; Klein *et al.*, 1991a) in response to the appropriate neurotrophin, indicating that p75 may be unnecessary for neurotrophic factor signalling. In contrast, other evidence suggested that p75 may be required for certain responses to neurotrophic factors. Transfection experiments using PC12 cells indicated that both *trk*A and p75 are necessary for the formation of functional, high-affinity NGF receptors (Hempstead *et al.*, 1989, 1991). Likewise, NGF-unresponsive medulloblastoma cells transfected with p75 cDNA exhibited both high- and low-affinity NGF binding and increased *c-fos* expression in response to NGF (Pleasure *et al.*, 1990). Also, neuroblastoma cells transfected with p75 cDNA extended neurites in response to NGF, indicating the importance of p75 for this function (Matsushima *et al.*, 1990). However, cells transfected with p75 cDNA lacking the region encoding the cytoplasmic domain showed only low-affinity binding with no increases in intracellular *c-fos* (Hempstead *et al.*, 1990) or tyrosine phosphorylation (Berg *et al.*, 1990) in response to NGF.

Several studies have been carried out to investigate the possible factors regulating p75 expression. NGF was shown to upregulate p75 mRNA levels in developing (Wyatt and Davies, 1993) and adult (Lindsay *et al.*, 1990) sensory neurons, central cholinergic neurons (Higgins *et al.*, 1989), and PC12 cells (Doherty *et al.*,

1988) *in vitro*. Tissue injury may affect p75 expression. Ernfors and co-workers (1989) found that axotomized motoneurons showed a dramatic increase in p75 expression. Other regulators of p75 expression were found to include bFGF, which increased p75 levels in PC12 cells (Doherty *et al.*, 1988) and in the MAH sympathoadrenal progenitor cell line (Birren *et al.*, 1990), and dexamethasone, which decreased low-affinity binding on PC12 cells (Tocco *et al.*, 1988) and p75 mRNA expression in MAH cells (Birren *et al.*, 1990).

The initial part of the present study of p75 was a comparison of p75 mRNA expression in chicken neurons that differ in their neurotrophic factor requirements. It was hoped that this would provide further insight into the role of the p75 receptor in forming functional receptors for the various neurotrophic factors. Four types of sensory neuron were studied: trigeminal mesencephalic (TMN) neurons which are proprioceptive neurons that are BDNF-and NT-3-responsive (Davies *et al.*, 1986a; Hohn *et al.*, 1990); dorsomedial trigeminal (DMTG) neurons which are small-diameter, neural crest-derived, cutaneous sensory neurons that respond to NGF (Davies and Lindsay, 1985); ventrolateral trigeminal (VLTG) neurons which are large-diameter, placode-derived, cutaneous sensory neurons that respond to BDNF (Davies *et al.*, 1986b); and jugular neurons which are like DMTG neurons in their morphology and function, and respond to NGF (Davies and Lindsay, 1985). It was thought that the varied neurotrophic requirements of these neurons might be reflected in their relative expression of p75 mRNA. As a further comparison, p75 mRNA levels were also measured in sympathetic chain neurons which are NGF-responsive (Chun and Patterson, 1977) and parasympathetic ciliary neurons which are not responsive to NGF, BDNF, or NT-3, but are CNTF-responsive (Barbin *et al.*, 1984). p75 mRNA levels were determined for these neurons at various stages of embryonic development, ranging from E10, when these neurons are dependent on neurotrophic factors for survival, through to E16.

The second part of this study of p75 investigated factors that might influence neuronal p75 mRNA levels *in vitro*. Immature E7 chicken sympathetic neurons were

used because these proliferate and survive in culture independently of neurotrophic factors (Ernsberger *et al.*, 1989a) and do not become NGF-responsive *in vitro* unless retinoic acid (RA, a derivative of retinol/vitamin A) is present in the culture medium (Rodriguez-Tébar and Rohrer, 1991). These characteristics were considered useful when studying factors that might regulate p75 mRNA levels, as such regulation could be linked with the induction of NGF-responsiveness. In agreement with this, Rodriguez-Tébar and Rohrer (1991) showed that RA not only induced NGF-dependency in E7 chicken sympathetic neurons *in vitro* but also increased the number of high-affinity receptors on these neurons.

In the present study, the effects of NGF, CNTF, stem cell factor (SCF), TGF-β1, -β2, -β3, and RA on *in vitro* p75 mRNA levels were investigated in E7 chicken sympathetic neurons. SCF is a ligand for the *c-kit* tyrosine kinase receptor which is widely expressed throughout the nervous system and is believed to influence migratory cells during development (Matsui *et al.*, 1990; Motro *et al.*, 1991; Keshet *et al.*, 1991). *In vitro*, SCF has been shown to support a proportion of NGF-responsive mouse (Hirata *et al.*, 1993) and chick (Carnahan *et al.*, 1994) dorsal root ganglion neurons. It does not enhance the survival of sympathetic, parasympathetic, or placode-derived sensory neurons (Carnahan *et al.*, 1994). The potential roles of TGF-βs include the control of embryonic development and cellular differentiation (review by Massagué *et al.*, 1994). Collins *et al.* (1991) found that membrane depolarization can promote neuronal survival by opening voltage-gated calcium channels. The effect of depolarization on p75 mRNA levels was studied by the addition of KCl to cultures. The effects of NGF, CNTF, and KCl on *in vitro* p75 mRNA levels in E14 sympathetic and DRG neurons were also studied as a comparison with the immature E7 sympathetic neurons.

Methods

In Vivo p75 Expression in Sensory, Sympathetic, and Parasympathetic neurons

The following ganglia were dissected, dissociated, and purified free of non-neuronal cells as described in Chapter 2: TMN, DMTG, VLTG, and jugular at E10, E12, and E14; lumbosacral paravertebral sympathetic chain at E10, E12, E14, and E16; and ciliary at E10, E12, and E16. Aliquots of approximately equal numbers of cells (as estimated using a haemocytometer) were centrifiged at 2,000 x g for 5 minutes and the supernatant removed prior to rapid freezing in liquid nitrogen and storage at -70°C. As required, cells were defrosted on ice and total RNA was extracted. p75 mRNA levels were determined using quantitative RT-PCR and these levels were standardized against levels of mRNA encoding the L27 ribosomal protein, also determined by RT-PCR. These procedures are described in Chapter 2.

In Vitro Regulation of p75 Expression in Sympathetic and Dorsal Root Ganglion Neurons

E7 and E14 chicken lumbosacral paravertebral sympathetic chain neurons and E14 chicken dorsal root ganglion neurons were dissected and placed in culture as described in Chapter 2. Dose responses were set up with E7 sympathetic neurons for SCF, NGF, and CNTF at concentrations of 0.001, 0.01, 0.1, 1, 5, 25, and 125 ng/ml and included control cultures with no added factors. E7 sympathetic neurons were also cultured with 35 mM additional KCl (F14 medium contains 5 mM KCl) and KCl plus 5 ng/ml CNTF. Cultures were incubated for 36 hours. A RA dose response was set up with E7 chicken sympathetic neurons using RA concentrations of 10^{-12}, 10^{-10}, 10^{-8}, 10^{-6}, and 10^{-4} M (according to Rodriguez-Tébar and Rohrer, 1991) and included control cultures with no RA added. Because the RA was dissolved in DMSO, neurons were also cultured in 1 µl DMSO/ml F14 (no RA) to check if the DMSO itself had any effect on p75 mRNA levels. These cultures were incubated for 48 hours. E7 chicken sympathetic neurons were cultured with TGF-β1 (50 ng/ml), TGF-β2 (20 ng/ml), and

TGF-β3 (20 ng/ml) and incubated for 48 hours.

E14 chicken sympathetic and DRG neurons were cultured with the following combinations of factors: NGF (10 ng/ml), CNTF (10 ng/ml), NGF plus CNTF (both at 10 ng/ml), additional KCl (35 mM), NGF plus additional KCl (10 ng/ml and 35 mM respectively). These cultures were incubated for 48 hours.

Treatments were set up in duplicate for both E7 and E14 neurons. Neuronal counts of all dishes were carried out prior to RNA extraction and RT-PCR quantitation of p75 mRNA levels as described in Chapter 2. Purified recombinant NGF, BDNF, CNTF, and TGF-βs were gifts from Genentech Inc. RA and KCl (tissue culture grade) were purchased from Sigma.

Results

Specificity and Reliability of Quantitation of p75 mRNA by RT-PCR

The levels of p75 mRNA in total RNA from purified neurons and neuronal cultures were measured using a quantitative RT-PCR technique. The p75 levels for purified neurons were expressed relative to mRNA levels for the ubiquitous and constitutively expressed L27 ribosomal protein, which were also determined by RT-PCR.

The target mRNAs in total RNA from neuronal samples were co-reverse transcribed with a slightly larger control RNA (prepared from p75 or L27 cDNAs) and co-amplified by PCR, as described more fully in Chapter 2. Co-reverse transcription and co-amplification of target mRNA and control RNA reduced the variables that affect reaction efficency. To determine the reliability of the RT-PCR technique, reactions were carried out using known quantities of target and control RNA transcripts. During the log-phase of the reaction, the ratio between the reaction products was identical to the initial ratio of target and control RNAs, regardless of the starting quantities of these RNAs (data not shown).

Examples of autoradiographs showing the separated target and control RT-PCR products for the p75 mRNA and L27 mRNA assays are given in figure 2. Total RNA

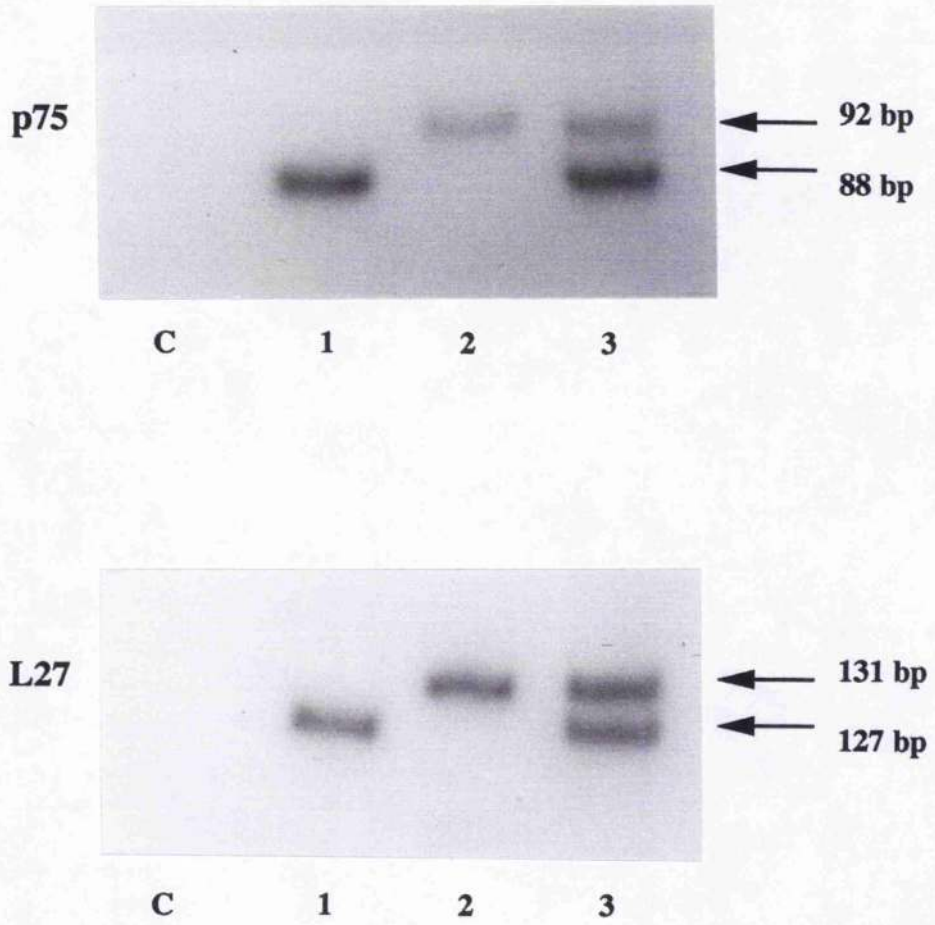


Figure 2 Reliability of the RT-PCR technique. Autoradiographs showing the products of RT-PCRs amplified with either p75-specific primers (p75) or L27-specific primers (L27). (1)Reactions containing total RNA from E10 chicken DMTG neurons, showing the 88 bp p75 mRNA and 127 bp L27 mRNA amplification products. (2) Reactions containing 50 fg of p75 control RNA template or 2 pg L27 control RNA template, showing 92 bp and 131 bp products respectively. (3) Reactions containing 50 fg of p75 control RNA template and 2 pg of the L27 control template plus total RNA from E10 DMTG neurons, showing both sets of the respective amplification products. (C) Control reactions showing no products; these contained the respective control RNA templates plus total RNA from E10 DMTG neurons but did not undergo the reverse transcription step.

that was not reverse transcribed gave no PCR products when amplified with either p75- or L27-specific primers, showing that the RNA sample contained no contaminating DNA. As little as 0.5 fg of p75 control RNA could be detected using the RT-PCR assay, allowing the measurement of target RNA in less than 10 neurons. The sensitivity of this technique has allowed for the measurement of p75 mRNA levels in low-density neuronal cultures.

In Vivo p75 Expression in Sensory, Sympathetic, and Parasympathetic Neurons

Figure 3 shows the *in vivo* p75 mRNA levels for embryonic chicken sensory, sympathetic, and parasympathetic neurons during development. For E10 sensory neurons, there was a significant difference between the p75 mRNA level of BDNF-responsive VLTG neurons and those of NGF-responsive DMTG and jugular neurons ($p<0.05$, t-test). However, the p75 mRNA level for BDNF- and NT-3-responsive TMN neurons is not significantly different from those of NGF-responsive DMTG and jugular neurons. This trend was observed throughout the period of development studied. Therefore, the data obtained show no clear-cut evidence that a relationship exists between p75 mRNA levels and neurotrophic factor requirement in sensory neurons at, or after, the time in embryonic development when they are dependent on neurotrophic factors for survival.

The p75 mRNA level for NGF-dependent E10 sympathetic neurons was similar to that for CNTF-dependent ciliary neurons. Both these values were significantly lower ($p<0.01$, t-test) than those for NGF-dependent DMTG and jugular neurons. The differences in sensory neuronal and sympathetic and parasympathetic neuronal p75 mRNA levels were maintained at E12 and also observed in the sensory and sympathetic neurons studied at E14. The overall levels of p75 expression increased slightly with embryonic age for all neurons studied except those of the TMN, in which there was a slight decrease. It is not clear what significance, if any, these fluctuations have for the function of p75 in sensory neurons. By E16, the p75 mRNA level for sympathetic

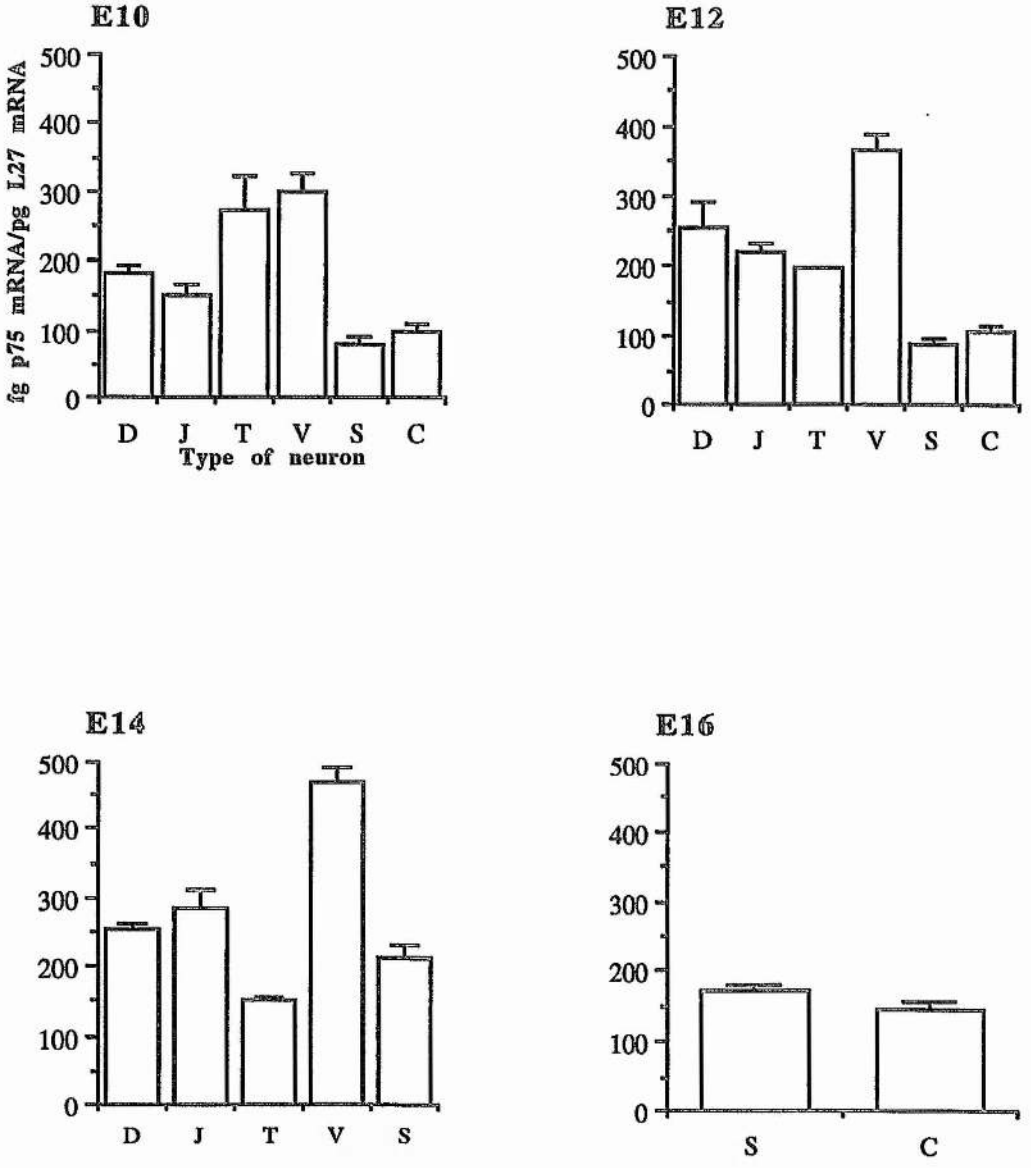


Figure 3 p75 mRNA expression relative to L27 mRNA in purified preparations of chicken sensory, sympathetic, and parasympathetic neurons at E10, E12, E14, and E16. D, DMTG neurons; J, jugular neurons; T, TMN neurons; V, VLTG neurons; S, sympathetic chain neurons; C, ciliary neurons. The graphs show the means of 4 samples for each neuronal type ± the standard error.

neurons was equivalent to the level for E10 NGF-responsive sensory neurons. This could indicate that the p75 receptor is more important in the development of sensory neurons or could merely represent the fact that sensory and sympathetic neurons start developing at different stages, sensory neurons being 'born' before sympathetic neurons.

In Vitro Regulation of p75 Expression in Sympathetic and DRG neurons

Figure 4 shows a phase contrast photomicrograph of E7 chicken sympathetic neurons surviving on a polyornithine-laminin substratum in the absence of neurotrophic factor (a control culture). The number of non-neuronal cells in the cultures was negligible. The data for *in vitro* regulation of p75 expression in E7 chicken sympathetic neurons are shown in figure 5. Part A of this figure shows the results for NGF, CNTF, and SCF dose responses. Increasing concentrations of NGF (added to a maximum of 125 ng/ml) failed to produce any significant change in p75 mRNA levels, which remained similar to the control level (no added NGF) of 1.13±0.092 fg/neuron. CNTF had a negligible effect on p75 mRNA levels. Even up to a concentration of 125 ng/ml, the p75 mRNA level remained similar to that of the control level (no added CNTF) of 1.31±0.085 fg/neuron. A similar result was obtained with the SCF dose response. Up to a maximum SCF concentration of 125 ng/ml, p75 mRNA levels remained similar to the control level of 1.29±0.205 fg/neuron. Neither CNTF nor SCF had an effect on neuronal survival and so did not appear to induce NGF dependency.

There was an increase in E7 sympathetic neuronal p75 mRNA level with increasing RA concentration *in vitro* (figure 5, part B). The level of p75 mRNA upregulation at a RA concentration of 10^{-4} M was significantly greater than the control p75 mRNA level ($p<0.02$, t-test). As no effect on neuronal survival was observed, it appeared that RA did not induce NGF dependency. The DMSO control (no RA) confirmed that the DMSO in which the RA was dissolved had no effect on neuronal survival or p75 expression.

The addition of TGF-β1, -β2, and -β3 to cultures of E7 sympathetic neurons

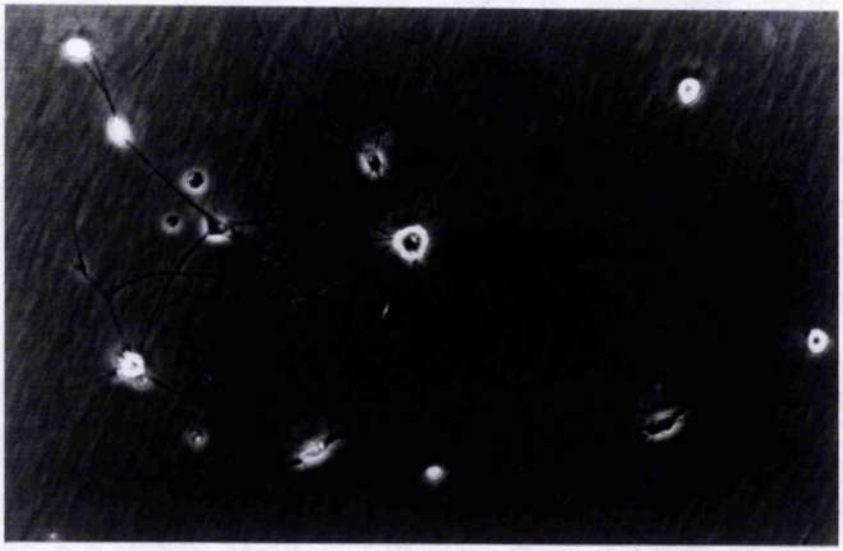


Figure 4 Phase contrast photomicrograph of E7 chicken sympathetic neurons surviving without neurotrophic factors on a polyornithine-laminin substratum. (A) A phase-bright E7 sympathetic neuron. (B) A more immature, phase-dark neuron. (Magnification x200.)

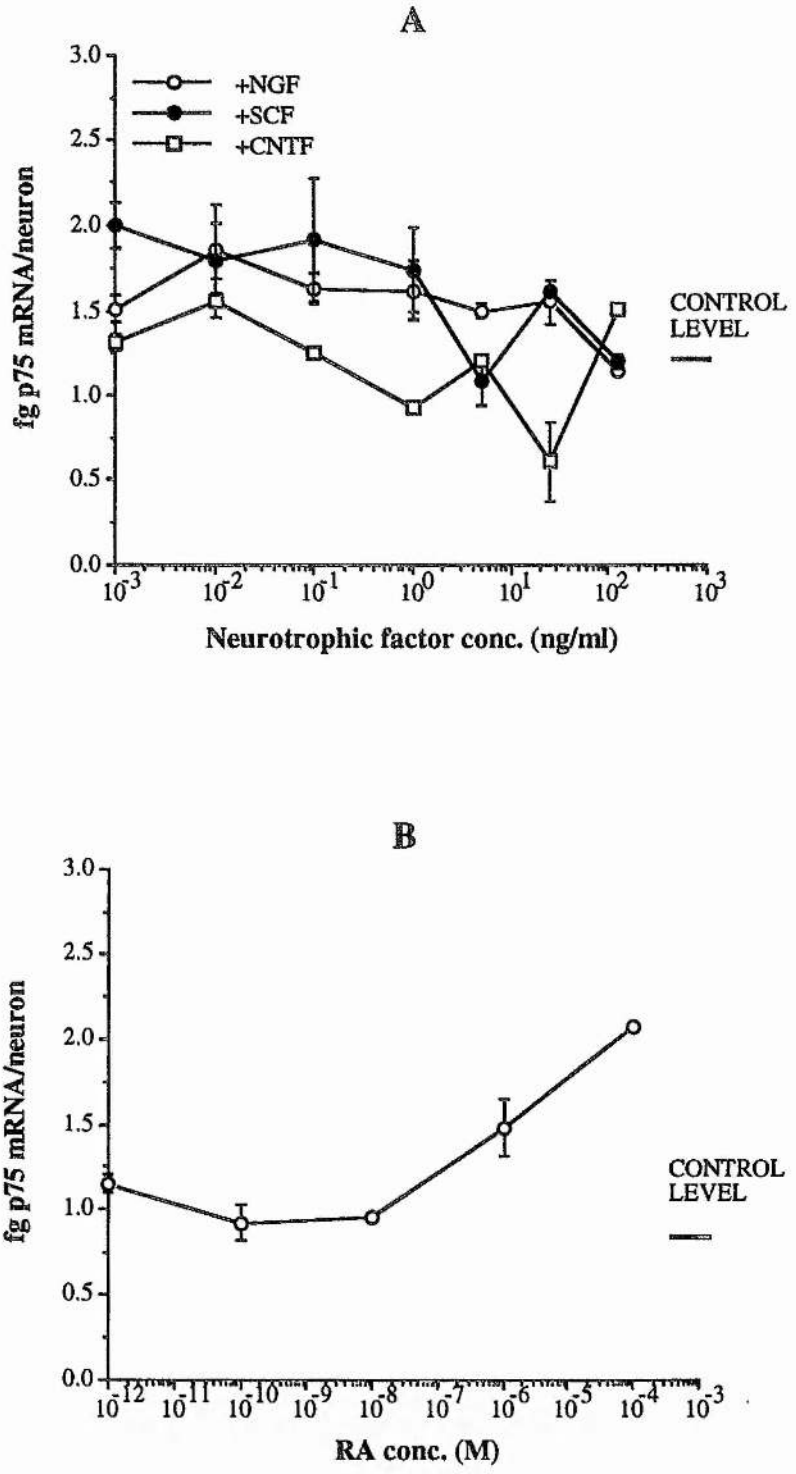
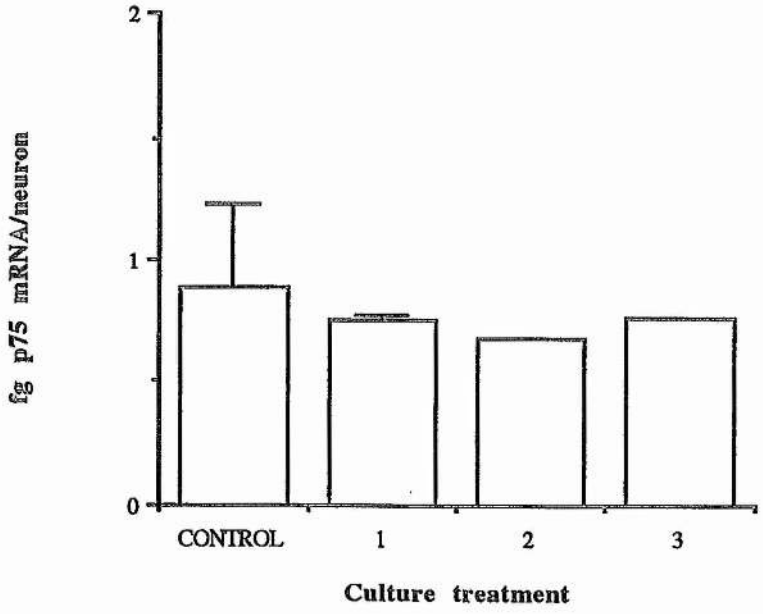


Figure 5 Regulation of *in vitro* p75 mRNA levels in E7 chicken sympathetic neurons. (A) NGF, CNTF, and SCF dose responses. The control p75 mRNA level (no factor added) is the mean control for all 3 dose responses, i.e., 1.24±0.095 fg/neuron (n = 6). (B) RA dose response. Control p75 mRNA level (no RA added) was 0.86±0.053 fg/neuron. p75 mRNA levels are expressed as fg/neuron. The graphs show the mean of two samples ± the standard error.

C



D

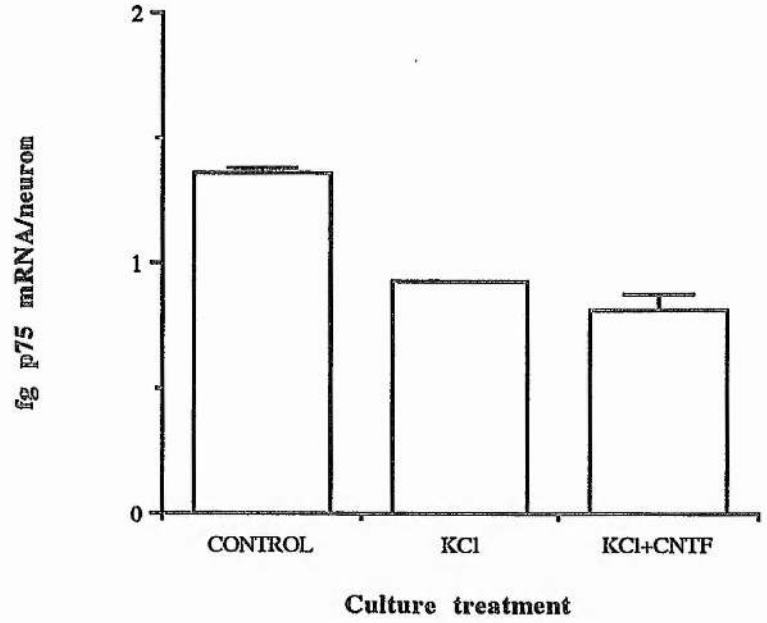


Figure 5 continued Regulation of *in vitro* p75 mRNA levels in E7 chicken sympathetic neurons. (C) Effect of TGF-βs. 1, TGF-β1 (50 ng/ml); 2, TGF-β2 (20 ng/ml); 3, TGF-β3 (50 ng/ml). (D) Effect of depolarization. KCl and/or CNTF were added at a concentration of 35 mM and 5 ng/ml respectively. p75 mRNA levels are expressed as fg/neuron. The graphs show the mean of two samples ± the standard error.

failed to significantly affect p75 mRNA levels; the level for each TGF-β remained similar to the control level of 0.88±0.035 fg/neuron (figure 5, part C). The TGF-βs had no effect on neuronal survival and so did not appear to induce NGF dependency.

Membrane depolarization of E7 sympathetic neurons, achieved by the addition of KCl to the cultures, produced a significant decrease in p75 mRNA level compared to the control level ($p<0.01$, t-test) (figure 5, part D). A similar decrease in p75 mRNA level was observed when KCl was added in combination with CNTF ($p<0.02$, t-test) (figure 5, part D). Depolarization appeared to have no effect on neuronal survival, either alone or in the presence of CNTF. It is unlikely that depolarization induced NGF dependency in these neurons.

p75 mRNA levels in NGF-dependent E14 sympathetic neurons treated in culture with NGF, CNTF, CNTF plus NGF, KCl, and NGF plus KCl showed no significant differences from that of the control culture treated with NGF (figure 6). For E14 DRG neurons treated in culture with NGF, CNTF, CNTF plus NGF, KCl, and NGF plus KCl, p75 mRNA levels were similar to that of the untreated control culture (figure 6).

Discussion

No conclusions for the selective role of the p75 receptor in modulating the function of particular neurotrophins could be made from studying the *in vivo* p75 mRNA levels of neurons with different neurotrophin requirements. There was no trend to suggest that p75 is more important in the functioning of any particular neurotrophin. More information is now available on the role of the low-affinity p75 receptor in modulating the effects of neurotrophins in sensory and sympathetic neurons. Perhaps one of the most revealing studies utilized mice with a null mutation in the p75 gene (Lee *et al.*, 1992). These mice showed reduced sensitivity to heat and painful stimuli. There were also some deficits in sympathetic innervation, particularly in the pineal gland and in a subset of the footpad sweat glands (Lee *et al.*, 1994a). *In vitro* studies using neurons from p75 null mutant mice have indicated that p75 modulates the effects of

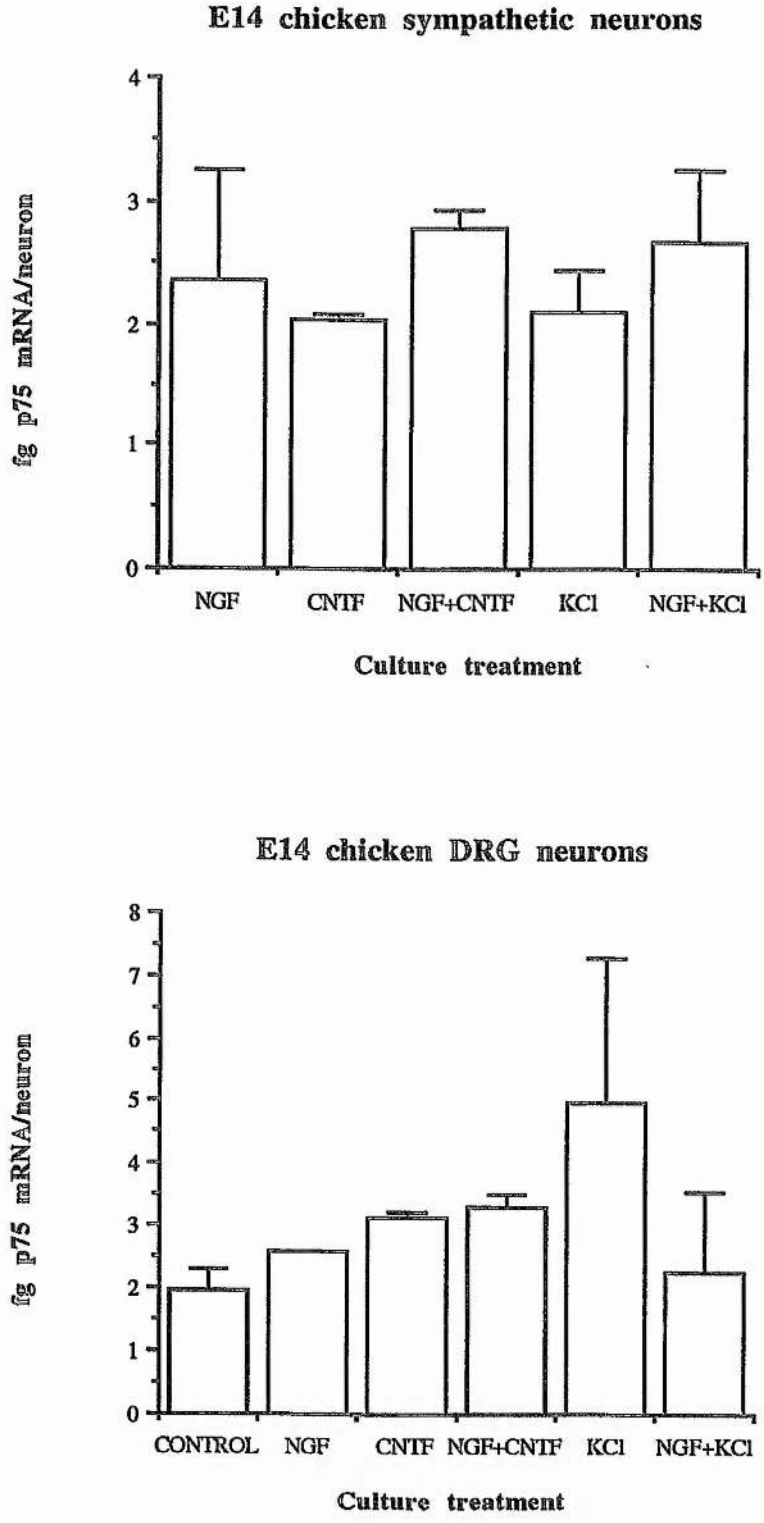


Figure 6 Regulation of *in vitro* p75 mRNA levels in E14 chicken sympathetic and DRG neurons. Effect of NGF (N, 10 ng/ml), CNTF (C, 10 ng/ml), additional KCl (K, 35 mM), NGF+CNTF, and NGF+KCl; CONT, untreated control. p75 mRNA levels are expressed as fg/neuron. The graphs show the mean of two samples ± the standard error.

NGF and accounts for the phenotype of null mutant mice. The NGF dose response of cutaneous sensory trigeminal neurons from null mutant embryos was shifted to a higher concentration compared to wild type neurons, while the BDNF and NT-4/5 dose responses of p75-deficient embryonic enteroceptive sensory nodose neurons remained unchanged (Davies *et al.*, 1993b). Studies comparing the NGF survival response of sympathetic neurons from p75 null mutant mice and wild type mice have shown that there is no difference in response prenatally, but postnatally p75-deficient neurons are less sensitive to NGF, suggesting that p75 plays an important role in sympathetic neurons late in their development (Davies *et al.*, 1993b; Lee *et al.*, 1994a). In the present study, the observation that p75 expression was lower in sympathetic neurons compared to sensory neurons raises the possibility that the p75 receptor might be more important in sensory neurons during the stages of development studied. To clarify this, a study of posthatching p75 expression should be carried out in sympathetic neurons to determine if p75 expression becomes elevated later in their development. The evidence clearly indicates that p75 plays a role in increasing neuronal sensitivity to NGF and indicates that both p75 and *trk*A receptors are required for maximum NGF responsiveness.

Interestingly, p75 mRNA was detected in parasympathetic ciliary neurons which do not respond to any member of the NGF family of neurotrophic factors. The p75 mRNA level detected in these neurons was similar to that of sympathetic neurons but much lower than those of the sensory neurons. Allsopp *et al.* (1993) have shown that the induced expression of *trk*A in ciliary neurons microinjected with the pCMX/*trk*A expression construct was insufficient to confirm an NGF survival response, even though *trk*A protein was detected on their cell bodies and neurites and their survival in medium containing CNTF was unaffected. In contrast, pCMX/*trk*A-microinjected TMN neurons (normally NGF-insensitive and BDNF- and NT-3-responsive) were supported in culture by NGF. It may be that, although ciliary neurons possess p75 receptors, they do not possess the intracellular signalling mechanisms which are initiated by *trk*A and p75 leading to survival.

In contrast to previously cited studies that have shown that NGF can upregulate p75 expression both *in vivo* (Wyatt and Davies, 1990 ; Higgins *et al.*, 1989; Miller *et al.*, 1991) and *in vitro* (Lindsay *et al.*, 1990; Doherty *et al.*, 1988), NGF failed to upregulate *in vitro* p75 expression in NGF-independent E7 chicken sympathetic neurons. Although NGF can upregulate p75 expression in more mature sympathetic neurons (Miller *et al.*, 1991), it is a possible that another factor is present which acts in conjunction with NGF to upregulate receptor expression in immature chicken sympathetic neurons *in vivo*. However, a recent study by Wyatt and Davies (1995) has shown that NGF can upregulate p75 expression in cultured immature mouse sympathetic neurons.

The effect of CNTF on E7 chicken sympathetic neurons has been characterized *in vitro* in the previously cited study by Ernsberger *et al.* (1989b). It was found to inhibit the *in vitro* proliferation of these neurons and affect differentiation by inducing the expression of vasoactive intestinal peptide. In the present study, CNTF was found to have no effect on *in vitro* p75 mRNA levels in these neurons. Hagg *et al.* (1992) have shown that, *in vivo*, CNTF prevents the degeneration of injured adult rat medial septum neurons (NGF rescues only the cholinergic neurons) and upregulates the p75 receptor in these neurons, as determined by immunostaining. CNTF may influence these central nervous system neurons differently compared to E7 chicken sympathetic neurons. However, it is possible that, *in vivo*, other factors may be acting in conjunction with CNTF and these interactions were not recreated *in vitro*.

SCF is the product of the *Sl* (Steel) locus and has been identified as the ligand for the *c-kit* tyrosine kinase receptor encoded by the *W* (Dominant white spotting) locus. Mice with mutations in both these loci develop abnormalities in three migratory cell lineages: neural crest-derived melanocytes, primordial germ cells, and haematopoietic stem cells. This results in loss of pigmentation, reduced fertility, and anaemia (Matsui *et al.*, 1990; Keshet *et al.*, 1991). Keshet *et al.* (1991) have shown that the developmental expression patterns of SCF and its receptor in skin and gonads indicate that the biological effect of SCF is to give a chemotactic signal to *c-kit*

expressing migratory cells. They have also shown that these genes are expressed in the nervous system and may play a role in the organization of the brain and neural tube. The primary function of SCF may be to provide a guidance mechanism for migratory cells during development. It is, therefore, not surprising that it failed to upregulate E7 sympathetic neuronal p75 expression, which could be considered as a maturational change. It has been shown that, unlike CNTF, SCF does not inhibit *in vitro* proliferation of these immature neurons (C.L. Stewart and A.M. Davies, personal communication).

RA is a naturally occurring metabolite of vitamin A that regulates cell growth and differentiation. Sidell (1982) observed that RA stimulated neurite outgrowth and inhibited proliferation in cultured human neuroblastoma cells (LA-N-1). Haskell *et al.* (1987) reported that RA increased the number of both high- and low-affinity NGF receptors in cultured human neuroblastoma cells (LA-N-1). They also reported that neurite outgrowth similar to that produced by NGF was observed in LA-N-1 cells, SY5Y neuroblastoma cells, and explanted chick DRG following treatment with RA. Scheibe and Wagner (1992) reported that RA stimulated p75 but not *trk*A expression in PC12 cells. Rodriguez-Tébar and Rohrer (1991) showed that RA induces the ability of immature chicken sympathetic neurons to survive in the presence of NGF and increases the number of high-affinity NGF receptors on these neurons. In the present study, RA was found to upregulate p75 expression in E7 chicken sympathetic neurons but did not have any detrimental effect on neuronal survival, which suggests that RA alone does not induce NGF responsiveness in these neurons. A number of RA receptors have been characterized (Giguère *et al.*, 1987; Brand *et al.*, 1988; Krust *et al.*, 1989; Rowe *et al.*, 1991). Strong expression of the RA receptor RXRα has been detected in the developing chick peripheral nervous system (Rowe *et al.*, 1991), so it is possible that RA may play an indirect role in inducing NGF responsiveness in immature sympathetic neurons.

Members of the TGF-β family have diverse roles in development, including the inhibition of cellular growth and differentiation and the control of hormone secretion

and immune function (Massagué *et al.*, 1994). The function of TGF-βs in the nervous system is not well understood. Nichols *et al.* (1991) have demonstrated that TGF-β mRNA is upregulated in the hippocampus after entorhinal lesioning and Lindholm *et al.* (1990) have shown that TGF-β1 stimulates NGF expression in cultured rat astrocytes. In addition, Chalazonitis *et al.* (1992) have reported that TGF-β1 and -β2 increase neuronal survival and expression of the peptide neurotransmitter substance P in cultures of neonatal rat DRG. They found that TGF-β1 enhanced the survival of cultured DRG neurons indirectly by acting on non-neuronal cells, although a direct effect on the neurons could not be ruled out. More recently, Buchman *et al.* (1994) have carried out *in vitro* studies of the effects of TGF-βs on NGF and NT-3 mRNA expression in dermal and epidermal cells from the maxillary territory of the embryonic mouse trigeminal ganglion. They found that, in E11 and E12 cultures, when the levels of NGF and NT-3 mRNAs are increasing *in vivo*, TGF-β1, -β2, and -β3 each increased the level of NGF mRNA but had no effect on the NT-3 mRNA level. In E13 cultures, when *in vivo* levels of NGF and NT-3 mRNAs reach a peak before falling dramatically in the case of NGF mRNA and gradually in the case of NT-3 mRNA, TGF-βs promoted further increases in the NGF mRNA level but caused a decrease in the NT-3 mRNA level. They also found that all three TGF-βs could be detected in the maxillary territory *in vivo*, before innervation by the earliest sensory axons and that their levels increased during the period of innervation. These observations suggest that TGF-βs may play a role in regulating neurotrophin gene expression in sensory neuron target fields. In the present study, the addition of TGF-β1, -β2, or -β3 to cultures of E7 chicken sympathetic neurons had no significant effect on p75 expression or neuronal survival.

It has been well documented that chronic depolarization increases the survival of many types of neuron in culture (review by Franklin and Johnson, Jr., 1992). Depolarization is usually achieved by elevating the level of K^+ ions in the culture medium and probably promotes survival by mimicking electrical activity that occurs *in vivo*, resulting in activation of voltage-gated calcium channels and raised intracellular

Ca^{2+}. Nishi and Berg (1981) have demonstrated that the survival response to depolarization in cultured chicken ciliary ganglion neurons could be blocked by the non-specific calcium channel blockers verapamil and Mg^{2+}. More specifically, Collins and Lile (1989) have shown that potent inhibitors of L-type calcium channels prevent depolarization, rescuing trophic factor-deprived chicken ciliary, sympathetic and DRG neurons. Conversely, Larmet *et al.* (1992) have shown that, *in vitro*, reduction of intracellular free calcium kills early neurotrophic factor-independent chicken nodose neurons but has no significant effect on the survival of older, BDNF-responsive neurons. The role of enhanced intracellular calcium in promoting neuronal survival is likely to involve the binding of free calcium to membrane-bound proteins which act as receptors to initiate a cascade of signalling events (review by Franklin and Johnson, Jr., 1992). Birren *et al.* (1992) have studied the effect of depolarization on *trk*A and p75 expression in MAH cells (an immortalized sympathoadrenal progenitor cell line). They found that depolarization induced expression of *trk*A but not p75 mRNA which was sufficient for a large proportion of the cells to develop and survive as neurons in NGF and FGF. However, depolarization does not induce *trk*A or p75 expression in normal mouse sympathetic neuroblasts *in vitro* (Wyatt and Davies, 1995). In the present study, depolarization was found to decrease *in vitro* p75 expression in E7 chicken sympathetic neurons. A similar decrease in p75 expression was also observed when these neurons were depolarized in the presence of CNTF, which has been shown to produce a maturational change by inhibiting neuronal proliferation and inducing cholinergic differentiation (Ernsberger *et al.*, 1989b). The significance of this downregulaton of p75 is unclear but it did not adversely affect neuronal survival.

p75 expression in NGF-dependent E14 chicken sympathetic neurons was unaffected *in vitro* by NGF, CNTF, NGF plus CNTF, KCl, and NGF plus KCl. It has been demonstrated that p75 expression in both mouse sympathetic neuroblasts and more mature mouse sympathetic neurons can be upregulated by NGF *in vitro,* while KCl has no effect (Wyatt and Davies, 1995). *In vitro* p75 mRNA levels in E14 DRG sensory neurons were also unaffected by NGF, CNTF, NGF plus CNTF, KCl, and

NGF plus KCl. Previous studies have shown that p75 expression in adult DRG can be upregulated by NGF both *in vivo* (Verge *et al.*, 1992) and *in vitro* (Lindsay *et al.*, 1990).

Conclusion

This study has shown that there are no clear-cut differences in the p75 mRNA levels of NGF-dependent cutaneous sensory DMTG and jugular neurons compared with BDNF- responsive TMN and VLTG neurons. Thus, although *in vitro* studies of the effect of the p75 null mutation have only revealed changes in the NGF dose response, not the BDNF dose response, this is not reflected in the relative levels of p75 mRNA (and possibly p75 protein) in NGF- and BDNF-responsive neurons. Perhaps p75 expression in BDNF-dependent neurons serves some other function. Likewise, p75 mRNA is expressed in ciliary neurons which have no known response to neurotrophins. It is possible that neurotrophins may have a role in the development of ciliary neurons that is not connected with survival.

The role of p75 regulation in the induction of NGF dependency in neurons was not established by this study. Although RA increased p75 mRNA levels in E7 chicken sympathetic neurons *in vitro*, it appeared to have no effect on neuronal survival. It is possible that p75 upregulation is one of a number of processes required to induce NGF responsiveness and not all of these can be recreated *in vitro*.

CHAPTER 4

Synthesis of BDNF by NGF-dependent Embryonic Sensory Neurons

Introduction

Substantial numbers of developing vertebrate sensory neurons die shortly after their axons reach their peripheral and central target fields (Oppenheim *et al.*, 1991). Experimental manipulation of target field size during this phase of development has been shown to influence the number of surviving neurons, indicating that target fields may play a major role in regulating neuronal survival (Davies, 1987). Further evidence shows that neurotrophic factors are responsible for the survival-promoting effects of sensory neuron target fields and are expressed in the peripheral and central target fields of these neurons during development (Davies *et al.*, 1987; Harper and Davies, 1990; Schecterson and Bothwell, 1992, 1994; Buchman and Davies, 1993; Buchman *et al.*, 1994; Hallböök *et al.*, 1993). Neurons can be rescued from naturally occurring cell death by the administration of exogenous neurotrophic factors (Levi-Montalcini and Angeletti, 1968; Hofer and Barde, 1988), while the use of blocking antibodies to these factors results in the elimination of neurons (Levi-Montalcini and Angeletti, 1968; Gaese *et al.*, 1994). Targeted null mutations in neurotrophic factors (Crowley *et al.*, 1994; Ernfors *et al.*, 1994a,b; Jones *et al.*, 1994) and their receptor tyrosine kinases (Klein *et al.*, 1993, 1994; Smeyne *et al.*, 1994) cause the loss of sensory neurons known to be supported by these factors *in vitro* (Davies, 1994a).

The expression of neurotrophic factors is not limited to the target fields of sensory neurons. BDNF mRNA has been detected in a proportion of embryonic dorsal root ganglion (DRG) neurons using *in situ* hybridization (Ernfors *et al.*, 1990b; Ernfors and Persson, 1991; Schecterson and Bothwell, 1992; Zhang *et al.*, 1994). This raises the possibility that BDNF could act on developing sensory neurons by an autocrine route. Strong evidence for an autocrine action of BDNF in sensory neurons has come from studies using antisense BDNF oligonucleotides to interfere with the production of

BDNF in cultured DRG neurons. This was initially carried out using DRG neurons which had been dissected out and cultured before their axons had reached their targets. At this stage, the neurons survive in isolation in defined medium without added neurotrophic factor and undergo a maturational change from small, spindle-shaped, phase-dark neurons with short neurites to larger, phase-bright, spherical neurons with long neurites. BDNF accelerates this maturational change, while antisense BDNF oligonucleotides have an inhibitory effect (Wright *et al.*, 1992), indicating that BDNF may promote maturation via an autocrine mechanism. The survival of early DRG neurons is unaffected by antisense oligonucleotides, suggesting that BDNF does not act by an autocrine mechanism to promote neuronal survival at this stage (Wright *et al.*, 1992). Recent studies using antisense BDNF oligonucleotides have also shown that BDNF acts by an autocrine route on adult DRG neurons (Acheson *et al.*, 1995). Like early DRG neurons that have not yet innervated their targets, adult DRG neurons survive independently of neurotrophic factors *in vitro* (Lindsay, 1988), but the BDNF autocrine loop at this stage is required for sustaining the survival of some of these neurons.

Now that BDNF has been shown to act on early and adult sensory neurons by an autocrine mechanism during developmental stages when these neurons survive independently of added neurotrophic factors (Wright *et al.*, 1992; Acheson *et al.*, 1995), it is important to determine whether a BDNF autocrine loop might also operate during the phase of naturally occurring neuronal death. This was addressed in part by measuring the levels of BDNF mRNA in highly purified preparations of different populations of cranial sensory neurons that depend on either NGF or BDNF for survival. During the latter half of embryonic development, neurons of the trigeminal mesencephalic nucleus (TMN) are supported by BDNF (Davies *et al.*, 1986a,b) but not NGF (Davies *et al.*, 1987), neurons of the dorsomedial part of the trigeminal ganglion (DMTG neurons) and jugular ganglion are supported by NGF and contain very few BDNF-responsive neurons, and neurons of the ventrolateral part of the trigeminal ganglion (VLTG neurons) are supported by BDNF and contain few NGF-responsive

neurons (Davies and Lindsay, 1985; Davies *et al.*, 1986a). In the chicken embryo, the number of neurons in the jugular ganglion peaks at E8 and decreases by half over the next 5 days (Hiscock and Straznicky, 1986) and the number of neurons in the TMN peaks at E9 and decreases by 80% over the next 5 days (Rogers and Cowan, 1974). Although there are no published data on the timing of neuronal death in the embryonic chicken trigeminal ganglion, these neurons are born over the same period of development as jugular and TMN neurons (between E2 and E7) (D'Amico and Noden, 1980; D'Amico, 1982; Hiscock and Straznicky, 1986). Thus, it is probable that all four populations will undergo naturally occurring neuronal death over much the same period of development. Hence, the expression of BDNF mRNA in these neurons was studied from E10 to E14. During this period, NGF-dependent neurons were found to express the highest levels of BDNF mRNA, while BDNF-dependent neurons expressed lower or undetectable levels of BDNF mRNA. This finding together with the demonstration that NGF-dependent sensory neurons synthesize and release biologically active NGF, suggests that, during the period of naturally occurring neuronal death, BDNF may act by a paracrine and not an autocrine route in certain sensory neurons.

Methods

Isolation and Purification of Neurons

The median part of the TMN, dorsomedial and ventrolateral poles of trigeminal ganglia, and the jugular ganglia were dissected from chicken embryos of 10 to 14 days incubation (E10 to E14). The ganglia were dissociated into neurons and these purified free of non-neuronal cells as described in Chapter 2.

Measurement of BDNF mRNA in Isolated Neurons

A quantitative RT-PCR technique (Wyatt and Davies, 1993) was used to measure the very low levels of BDNF mRNA in purified neuronal samples as described in Chapter 2.

To compare the relative level of BDNF mRNA in different kinds of sensory

neurons, the level of L27 mRNA, which codes for a ubiquitous, constitutively expressed ribosomal protein, was measured in RNA samples from purified neurons by quantitative RT-PCR. The level of BDNF mRNA in samples could then be expressed relative to the level of L27 mRNA in these samples. The RT-PCR protocol for measuring L27 mRNA levels is given in Chapter 2.

Neuronal Cultures

To study the survival response of purified E10 TMN, DMTG, VLTG, and jugular neurons to BDNF and NGF, these neurons were plated in 35 mm tissue culture dishes (Nunc) that had been pre-coated with P-ORN (0.5 mg/ml in 0.15 M borate buffer, pH 8.4, overnight) and laminin (20 μg/ml in F14 medium, 4 hours). The neurons were grown in 2 ml of Ham's F14 medium plus 10% heat-inactivated horse serum (HIHS) supplemented with either 5 ng/ml NGF or 5 ng/ml BDNF, and were incubated at 37°C in a humidified 4% CO_2 incubator. The tissue culture procedure is described in more detail in Chapter 2. Six to twelve hours after plating, the number of attached neurons within a 12 x 12 mm square in the centre of each dish was counted. The number of surviving neurons in this same area was counted after 48 hours incubation and is expressed as a percentage of the number of attached neurons at 6 to 12 hours.

To determine if BDNF mRNA-expressing neurons synthesize and release BDNF, BDNF mRNA-expressing DMTG neurons were co-cultured with BDNF-dependent TMN neurons. High density cultures of purified E10 DMTG neurons were first established in 11 mm diameter tissue culture wells (Greiner) pre-coated with P-ORN and laminin. The neurons (approximately 5,000 per well) were grown in 100 ml of F14 medium with 10% HIHS and 5 ng/ml NGF (to promote their survival). After 24 hours incubation, purified E10 TMN neurons were plated at lower density (between 300 and 500 neurons per well). To distinguish the TMN neurons from the DMTG neurons, the former were labelled prior to plating with DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, from Molecular Probes) (Honig and Hume,

1986) by incubating them in a 40 μg/ml solution of DiI in Ham's F14 medium containing 10% HIHS for 1 hour at 37°C in a bacteriology grade plastic petri dish. The neurons were then washed three times in F14 plus 10% HIHS; the neurons were pelleted by centrifugation (2,000 g for 5 minutes) to remove the medium between washes. The labelled neurons were visualized with an inverted fluorescent microscope using rhodamine filters after a further 24 hours in culture. TMN neurons were also cultured alone with 5 ng/ml BDNF. A TrkB-IgG immunoadhesin (Shelton *et al.*, 1995), which binds and inhibits the action of BDNF, was added to TMN/DMTG neuronal co-cultures at a concentration of 50 ng/ml to determine if the survival-promoting effect of the DMTG neurons was due to their synthesizing and secreting BDNF. In addition, TMN neurons were cultured with 5 ng/ml BDNF plus 50 ng/ml TrkB-IgG. The TrkB-IgG was a gift from Dave Shelton of Genentech Inc and the purified recombinant NGF and BDNF used in all these neuronal culture studies were gifts from Gene Burton of Genentech Inc.

Results

BDNF mRNA Expression by Different Kinds of Sensory Neurons

Quantitative RT-PCR was used to measure BDNF mRNA levels in total RNA extracted from >95% pure preparations of DMTG, jugular, TMN, and VLTG neurons obtained from E10, E12 and E14 chicken embryos. An example of an autoradiograph showing the separated RT-PCR products for the BDNF mRNA assay is given in figure 7. The levels of BDNF mRNA in different total RNA samples were standardized against the levels of mRNA encoding the ubiquitous, constitutively expressed L27 ribosomal protein, also determined using RT-PCR. The sensitivity and accuracy of the competitive RT-PCR technique used in these studies has been documented previously (Wyatt and Davies, 1993). Figure 8 shows that, at each age, BDNF mRNA levels were highest in DMTG and jugular neurons and increased approximately two-fold between E10 and E14. Lower levels of BDNF mRNA were expressed in VLTG neurons and BDNF mRNA was undetectable in TMN neurons.

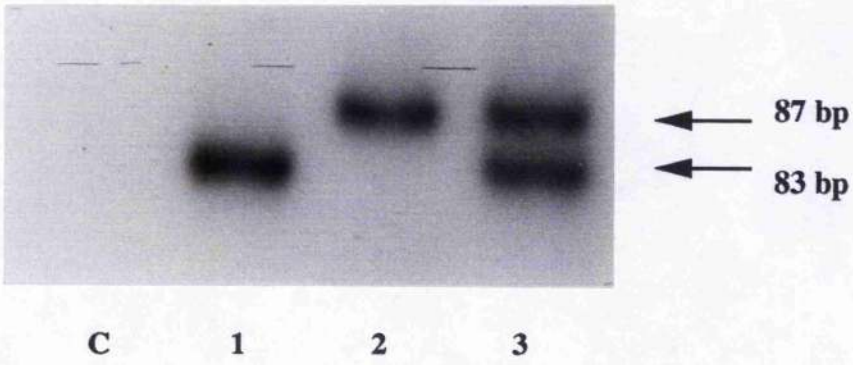


Figure 7 Autoradiograph showing the products of RT-PCRs amplified with BDNF-specific primers. (1) Reaction containing total RNA from purified E10 chicken DMTG neurons showing the 83 bp amplification product. (2) Reaction containing 0.5 fg of control RNA template, showing the 87 bp amplification product. (3) Reaction containing 0.5 fg control RNA template plus total RNA from purified E10 chicken DMTG neurons, showing both amplification products. (C) Control reaction showing no products; these contained the control RNA template plus total RNA from E10 DMTG neurons but did not undergo the reverse transcription step.

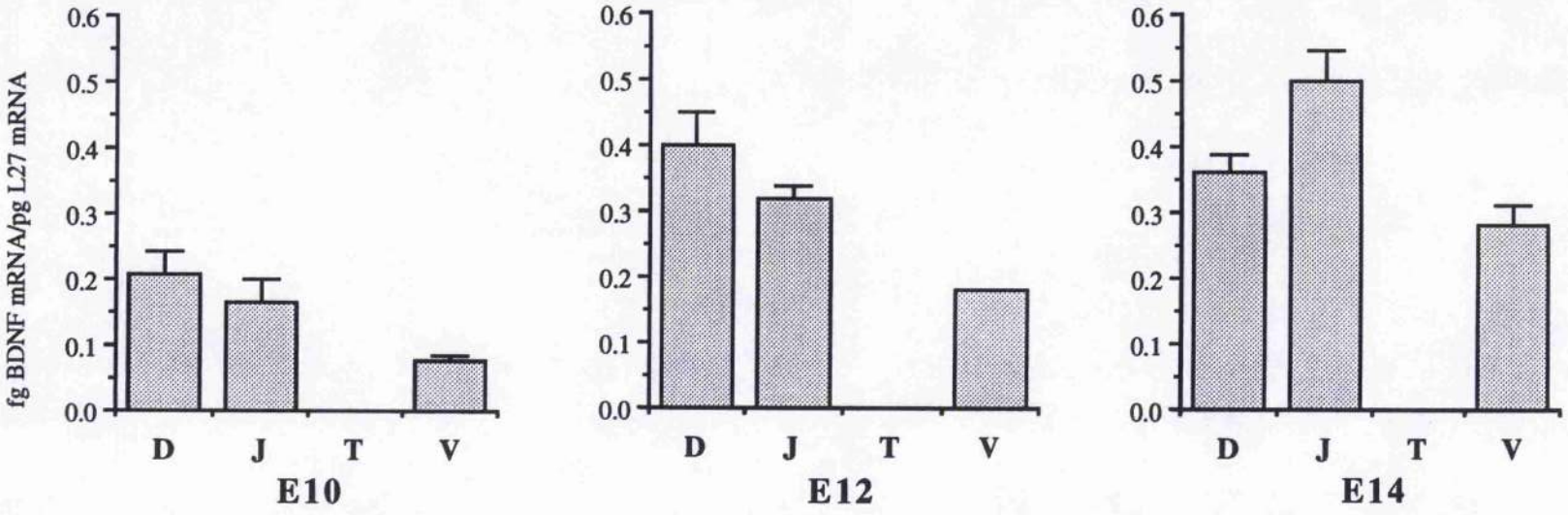


Figure 8 Bar chart showing BDNF mRNA levels relative to L27 mRNA in purified DMTG (D), jugular (J), TMN (T), and VLTG (V) neurons from E10, E12, and E14 chicken embryos. The mean and standard error of measurements of 4 separate samples of each kind of neuron at each age point are shown.

The survival responses of DMTG, jugular, TMN, and VLTG neurons to NGF and BDNF are shown in figure 9. The majority of DMTG and jugular neurons were supported for 48 hours by NGF, with only a small population of between 5 and 10% responding to BDNF. Conversely, the majority of TMN and VLTG neurons were supported by BDNF. NGF did not support the survival of any TMN neurons and promoted the survival of about 10% of VLTG neurons. These findings are similar to previously reported survival responses of DMTG, VLTG, and TMN neurons to NGF and BDNF (Davies *et al.*, 1986a,b, 1987).

Thus, during the middle phase of embryonic development when naturally occurring neuronal death takes place in populations of cranial sensory neurons (Rogers and Cowan, 1974; Hiscock and Straznicky, 1986), BDNF mRNA is expressed predominantly in populations of NGF-dependent sensory neurons. The BDNF-dependent TMN neurons, which contain no NGF-responsive neurons, do not express BDNF mRNA, while the VLTG neurons, which contain a minor set of NGF-responsive neurons, express lower levels of BDNF mRNA than DMTG and jugular neurons.

BDNF Synthesis by NGF-Dependent Neurons

Whether or not BDNF mRNA-expressing, NGF-dependent sensory neurons synthesize and release biologically active BDNF was determined by co-culturing NGF-dependent DMTG neurons with BDNF-dependent TMN neurons to see if the former could promote the survival of the latter without BDNF in the medium. Before plating, the TMN neurons were labelled with DiI, a fluorescent, lipophilic, carbocyanine dye (Honig and Hume, 1986) so that they could be identified in the co-cultures. Figure 10 shows how DiI labelling allowed TMN neurons to be easily identified in TMN/DMTG neuronal co-cultures. Since TMN neurons do not respond to NGF (Davies *et al.*, 1987), the survival of DMTG neurons in co-cultures could be promoted by adding NGF to the culture medium without affecting the survival of TMN neurons.

All TMN neurons grown alone in medium supplmented with NGF were dead

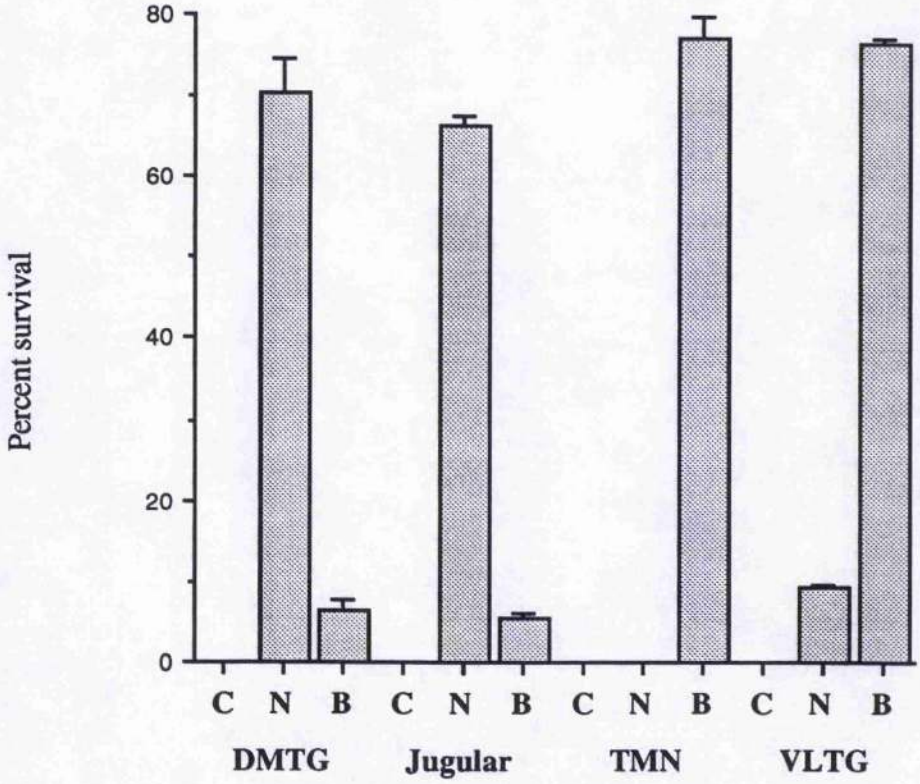


Figure 9 Bar chart showing the percent survival of E10 DMTG, jugular, TMN, and VLTG neurons after 48 hours in control cultures (C) and cultures supplemented with 5 ng/ml NGF (N) and 5 ng/ml BDNF (B). The mean and standard error of the neuronal counts for three separate culture dishes are shown.

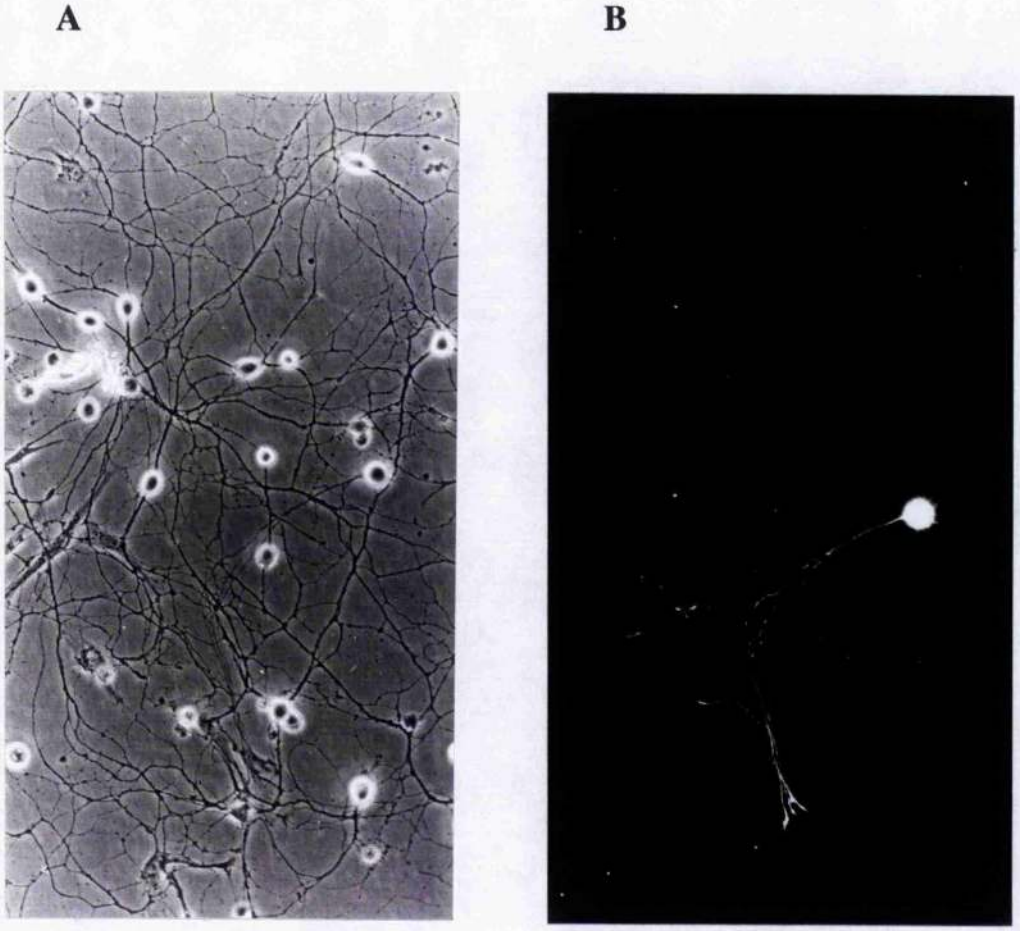


Figure 10 Photomicrographs of co-cultures of TMN and DMTG neurons. (A) Phase contrast view showing all neurons and their processes in the field. (B) The same field as in (A) viewed by rhodamine epifluorescence showing DiI-labelled TMN neuron. The remaining neurons (DMTG) are not fluorescent.

after 48 hours of incubation (data not shown), whereas approximately 50% of TMN neurons were still surviving when co-cultured with DMTG neurons in the absence of added BDNF (figure 11). This was almost equivalent to the percentage of TMN neurons that survived alone in BDNF-supplemented medium (figure 11). To ascertain whether the survival-promoting effect of DMTG neurons on TMN neurons was due to the synthesis and release of BDNF, a TrkB-IgG immunoadhesin (Shelton *et al.*, 1995) was added to the culture medium to bind and inhibit the action of BDNF. In cultures of TMN neurons grown alone in medium containing 5 ng/ml BDNF, TrkB-IgG at a concentration of 50 ng/ml reduced neuronal survival from 67% to 8% (figure 11). The same level of TrkB-IgG did not affect the number of E10 DMTG neurons surviving in medium containing 5 ng/ml NGF (data not shown). TrkB-IgG reduced the survival of TMN neurons growing in DMTG/TMN neuronal co-cultures from 51% to 22% (figure 11). This is strong evidence that the survival-promoting effect of DMTG neurons on TMN neurons was at least partly due to the synthesis and release of BDNF by DMTG neurons.

Discussion

Since BDNF is expressed in a proportion of embryonic DRG neurons (Ernfors *et al.*, 1990b; Ernfors and Persson, 1991; Schecterson and Bothwell, 1992; Zhang *et al.*, 1994) and studies have shown that BDNF acts on sensory neurons via an autocrine mechanism at stages in their development when they are independent of target-derived neurotrophic factors (Wright *et al.*, 1992; Acheson *et al.*, 1995), a BDNF autocrine mechanism might also exist during the phase of naturally occurring neuronal death. This would make the process of neuronal selection more complex than first proposed by the classic neurotrophic theory which states that the survival of sensory neurons during this period is promoted by only target-derived neurotrophic factors. If BDNF were to promote survival via an autocrine action, it would compromise the neuronal selection process by target fields.

As the highest levels of BDNF mRNA are expressed in NGF-dependent DMTG

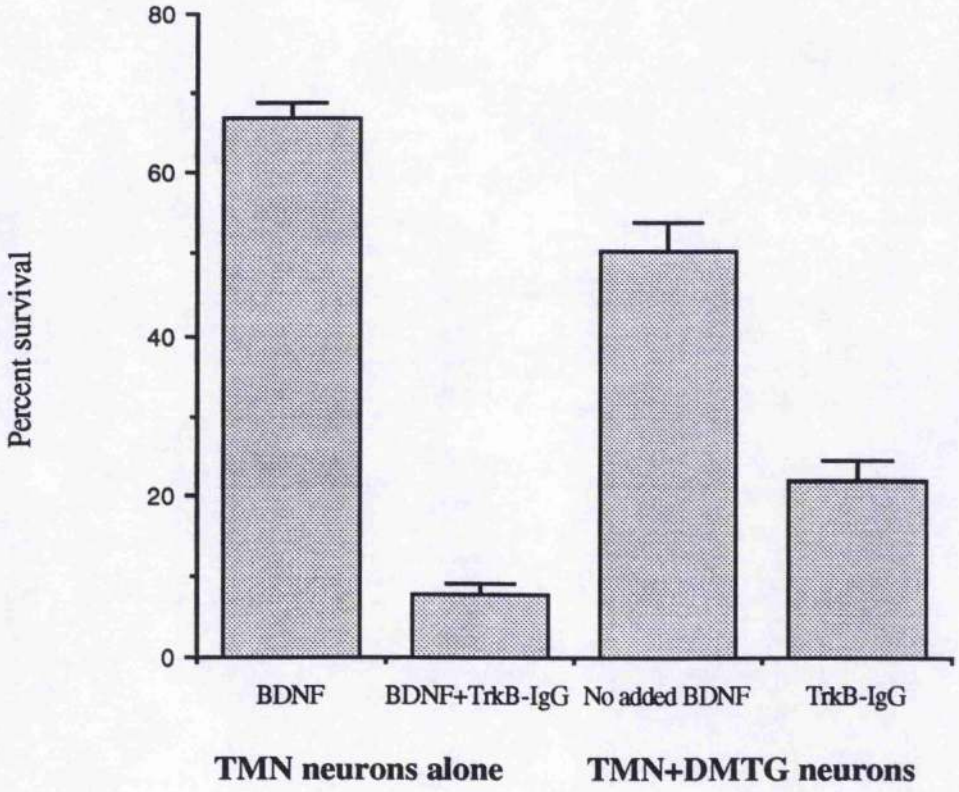


Figure 11 Bar chart showing the percent survival of E10 TMN neurons cultured alone or co-cultured with a ten-fold greater number of E10 DMTG neurons. All cultures were supplemented with 5 ng/ml NGF. In addition, TMN neurons were cultured with either 5 ng/ml BDNF or 5 ng/ml BDNF plus 50 ng/ml TrkB-IgG. TMN/DMTG neuronal co-cultures were also grown with or wthout 50 ng/ml TrkB-IgG. The mean and standard error of three separate experiments, each set up in duplicate, are shown.

and jugular neurons during the period of naturally occurring neuronal death, it is unlikely that BDNF acts via an autocrine mechanism to promote the survival of these cutaneous sensory neurons during this stage of their development. Likewise, the fact that BDNF mRNA expression could not be detected in BDNF-dependent TMN neurons during the period of naturally occurring neuronal death suggests that BDNF does not act by an autocrine mechanism in these proprioceptive neurons during this stage. A lower level of BDNF mRNA expression was found for the primarily BDNF-dependent VLTG neurons, raising the possibility that BDNF may act by an autocrine route in these cutaneous sensory neurons. However, this could be ruled out by the fact that the amount of BDNF produced was insufficient to promote the survival of E10 VLTG neurons grown without BDNF. Moreover, it has been shown that, at sub-saturating levels of BDNF, the difference in the survival responses of E10 TMN and VLTG neurons are not sufficient (L. Pinon and A. M. Davies, unpublished findings) to account for any enhancement in VLTG neuronal survival by the autocrine action of BDNF.

The finding by this study that NGF-dependent embryonic sensory neurons synthesize sufficient BDNF to promote the survival of co-cultured BDNF-dependent neurons suggests a possible role for NGF-dependent sensory neurons in the maintenance of BDNF-responsive neurons *in vivo*. It is possible, for example, that BDNF-producing, NGF-dependent sensory neurons synapse with BDNF-dependent second order sensory neurons in the central nervous system. DRG comprise both NGF- and BDNF-responsive neurons which would make a paracrine action between neuronal cell bodies in the intact developing ganglion a theoretical possibility. In practice, however, this is unlikely to be the case because it would interfere with the target selection of neurons during the phase of naturally occurring neuronal death.

Conclusion

The work carried out in this study has revealed an important insight into the neurotrophic interactions between sensory neurons. Further studies are required to

determine, firstly, the extent to which neurotrophic factors are transported to and released from specific parts of factor-producing neurons and, secondly, what mechanisms might regulate these processes. This information will clearly be important in resolving the function and sites of action of BDNF produced by developing sensory neurons.

CHAPTER 5

Timing and Regulation of *trk*B and BDNF mRNA Expression in Placode-Derived Sensory Neurons and Their Targets

Introduction

Neurons in the developing vertebrate peripheral nervous system require a supply of neurotrophins from the tissues they innervate in order to survive (Davies, 1994c). When sensory and sympathetic neurons are placed in culture before they have innervated their targets, they survive and extend axons in the absence of added neurotrophins (Davies and Lumsden, 1984; Coughlin and Collins, 1985; Ernsberger and Rohrer, 1988; Davies, 1989; Ernsberger *et al.*, 1989a) and only become dependent on a particular neurotrophin for survival at the stage when their axons would normally encounter their targets *in vivo* (Davies and Lumsden, 1984; Vogel and Davies, 1991; Buchman and Davies, 1993).

In vitro studies of different populations of cranial sensory neurons have provided insight into how the timing of neurotrophin dependence is regulated (Davies, 1987, 1994b). In the chicken embryo, several populations of these neurons are derived from ectodermal placodes (D'Amico and Noden, 1983), are 'born' during the same period of development (D'Amico, 1982), and start extending axons to their peripheral and central targets at approximately the same time, but differ in the distance their axons have to grow to reach these targets (Davies, 1989). The two extremes are the neurons of the vestibular ganglion, which lie close to the hindbrain and innervate nearby targets in the developing otic vesicle, and the neurons of the nodose ganglion, which are located some distance away from the hindbrain and innervate distant targets in the thorax and abdomen, such as the heart (Davies, 1994b). When placed in culture at the stage when the first axons are beginning to grow towards their central and peripheral targets, vestibular neurons die rapidly in the absence of neurotrophins whereas nodose neurons die much more slowly. Hence, early vestibular neurons start responding to

BDNF with enhanced survival before nodose neurons (Vogel and Davies, 1991). Because these experimental results were obtained in very low density cultures, it is likely that early cranial sensory neurons are individually programmed to survive for a specific length of time before they become dependent on BDNF for survival (i.e., these survival differences are intrinsic properties of the neurons).

Although the timing of BDNF dependence appears to be controlled primarily by an intrinsic programme, there is some evidence that the onset of dependence can be accelerated by BDNF itself. Early nodose neurons normally begin responding to BDNF with enhanced survival after about 72 hours in culture. However, if these neurons are transiently exposed to BDNF between 48 and 72 hours, they die more rapidly and to a greater extent after 72 hours following BDNF deprivation than neurons that were not exposed to BDNF (Vogel and Davies, 1991).

BDNF exerts its effects on neurons by binding to the *trk*B receptor, a member of the *trk* family of tyrosine kinase receptors (Squinto *et al.*, 1990; Berkemeier *et al.*, 1991; Klein *et al.*, 1991b, 1992; Soppet *et al.*, 1991; Ip *et al.*, 1992). In addition to catalytic *trk*B receptors that possess a kinase domain, alternative splicing generates *trk*B variants that lack the tyrosine kinase domain (Klein *et al.*, 1990a,b), although the function of these non-catalytic receptors is unclear. BDNF, like other neurotrophins, also binds to the common neurotrophin receptor, p75. However, the function of p75 in mediating or modulating the BDNF survival response, if any, is unclear. Although p75 null mutant mice have defective innervation of tissues by some NGF-responsive sensory and sympathetic neurons (Lee *et al.*, 1992, 1994a) and p75-deficient embryonic sensory and postnatal sympathetic neurons are less responsive to NGF than wild type neurons (Davies *et al.*, 1993b; Lee *et al.*, 1994b), the dose response of p75-deficient nodose neurons to BDNF is normal (Davies *et al.*, 1993b). Two issues have yet to be studied. Firstly, how differences in the timing of the BDNF survival response are related to the expression of catalytic *trk*B and, secondly, whether the effect of BDNF on accelerating BDNF dependence in early sensory neurons is related to changes in *trk*B expression. In an attempt to address these issues, the timing and

regulation of catalytic *trk*B mRNA expression was studied in developing sensory neurons. p75 mRNA expression in BDNF-dependent neurons was also studied to obtain potentially useful information for comparison with p75 mRNA expression in NGF-responsive neurons.

Another important but poorly understood issue is the extent to which BDNF acts on developing sensory neurons by an autocrine route (Davies and Wright, 1995). Many embryonic dorsal root ganglion (DRG) neurons express BDNF mRNA (Ernfors *et al.*, 1990b; Ernfors and Persson, 1991; Schecterson and Bothwell, 1992; Zhang *et al.*, 1994) and antisense BDNF oligonucleotides inhibit an early maturational change in many cultured early DRG neurons before they become dependent on added neurotrophins for survival (Wright *et al.*, 1992). Similar experiments on adult DRG neurons, which like very early sensory neurons survive independently of neurotrophins in culture (Lindsay, 1988), have shown that antisense BDNF oligonucleotides reduce the number of surviving neurons by 35% (Acheson *et al.*, 1995), suggesting that BDNF acts by an autocrine route to promote the survival of a subset of these neurons. The BDNF autocrine loop in early DRG neurons, however, does not seem to play a role in neuronal survival because antisense BDNF oligonucleotides do not affect the number of surviving neurons (Wright *et al.*, 1992). Studying when *trk*B is expressed in developing sensory neurons would provide additional information on when BDNF autocrine loops are potentially capable of influencing neuronal development.

The present study focused on the two populations of cranial sensory neurons that have the most extreme differences in the duration of neurotrophin independence, those of the vestibular and nodose ganglia. Nodose neurons with their particularly lengthy phase of neurotrophin independence were especially useful for studying the timing and regulation of *trk*B expression. Because only very small numbers of cranial sensory neurons were available from early chicken embryos for these studies a reliable, quantitative, competitive reverse transcription-polymerase chain reaction (RT-PCR) technique was used (Wyatt and Davies, 1993) to measure the level of *trk*B mRNA encoding functional *trk*B receptors that possess a kinase domain. Results obtained in

the present study show that *trk*B mRNA is expressed with a different time course in vestibular and nodose neurons that accords with the different timing of the BDNF survival response in these neurons. Moreover, the results on the regulation of *trk*B mRNA expression in cultured neurons suggest that BDNF's modulation of when these neurons acquire neurotrophin dependence may be mediated by changes in *trk*B expression. It was also shown that a BDNF autocrine loop cannot operate in at least some populations of sensory neurons during the earliest stages of neurotrophin independent survival.

Materials and Methods

Embryonic Tissues

Tungsten needles were used to dissect the nodose and vestibular ganglia, heart, otic vesicle and hindbrain from staged (Hamburger and Hamilton, 1951) chicken embryos. General dissection procedures are given in Chapter 2. Adherent mesenchymal tissue was removed from the hindbrain and otic vesicle by incubating these tissues with 10 mg/ml dispase (Boehringer-Mannheim, grade II) in HEPES-buffered (20 mM) Hanks' balanced salt solution for 5 minutes at 25^{o}C. Tissues for RNA extraction were frozen and stored at -70^{o}C.

Neuronal Cultures

Stage 19 nodose ganglia were trypsinized for 8 minutes and dissociated to give a single cell suspension as described in Chapter 2. The cells were plated at a density of 1,000-1,500 neurons per dish in 60 mm plastic tissue culture dishes (Nunc) that had been pre-coated with P-ORN (0.5 mg/ml in 0.15 M borate buffer, overnight) and laminin (20 μg/ml for 4 hours). The neurons were incubated at 37^{o}C in a humidified 4 % CO_2 incubator in 4 ml of Ham's F14 supplemented with penicillin (60 mg/l), streptomycin (100 mg/l), 10% heat-inactivated horse serum (HIHS) and 5% heat-inactivated fetal calf serum (HIFCS, Gibco BRL). The neurons were grown with or without purified recombinant BDNF (gift of John Winslow and Gene Burton,

Genentech Inc) at a concentration of 5 ng/ml. Chapter 2 gives further details of the neuronal culture procedure.

Measurement of Specific mRNA Transcripts

A quantitative, competitive, RT-PCR technique (Wyatt and Davies, 1993) was used to measure the levels of mRNAs encoding BDNF, catalytic *trk*B, p75 , and L27 in total RNA from tissues and cultured neurons. Details of the RNA extraction procedure and RT-PCR assays are given in Chapter 2.

Results

Expression of *trk*B mRNA in Developing Nodose and Vestibular Ganglia

The levels of mRNA encoding full-length, catalytic *trk*B in the vestibular and nodose ganglia of chicken embryos during the early stages of axonal outgrowth and target encounter were determined using RT-PCR (Wyatt and Davies, 1993). An example of an autoradiogram showing the separated PCR products is shown in figure 12. Figure 13 shows the level of *trk*B mRNA in vestibular and nodose ganglia from stages 17 to 24 (Hamburger and Hamilton, 1951). To compare the relative levels of *trk*B mRNA in these ganglia, quantitative RT-PCR was used to measure the mRNA level of L27, a ubiquitous, constitutively expressed ribosomal protein, in RNA samples used for measuring *trk*B mRNA. At stage 17, *trk*B mRNA was not detected in either ganglion. *trk*B mRNA was first detected in the vestibular ganglion at stage 18 and at stage 21 in the nodose ganglion. The earlier appearance of *trk*B mRNA in the vestibular ganglion accords with the shorter period of neurotrophin independence and earlier response of these neurons to BDNF compared with nodose neurons (Vogel and Davies, 1991).

Expression of p75 mRNA in Developing Nodose and Vestibular Ganglia

Although the function of p75 in mediating or modulating the BDNF survival response is unclear, the level of p75 mRNA was measured in vestibular and nodose

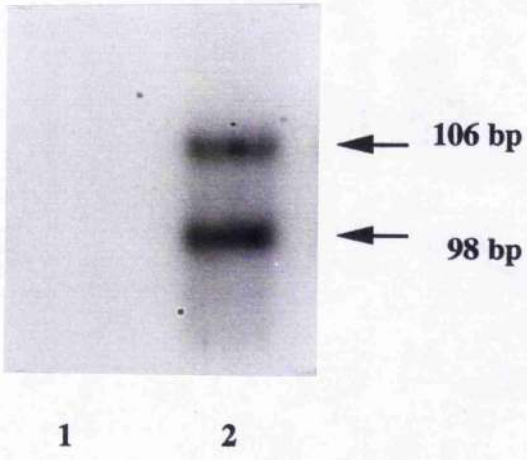


Figure 12 Autoradiograph showing the products of RT-PCRs amplified with primers specific for full-length chicken *trk*B. (1) Control reaction showing no amplification products; this contained 1 fg of the control RNA template plus total RNA from stage 24 nodose ganglia but did not undergo the reverse transcription step. (2) Reaction containing 1 fg of the control RNA template plus total RNA from stage 24 nodose ganglia, showing the 106 bp and 98 bp amplification products respectively.

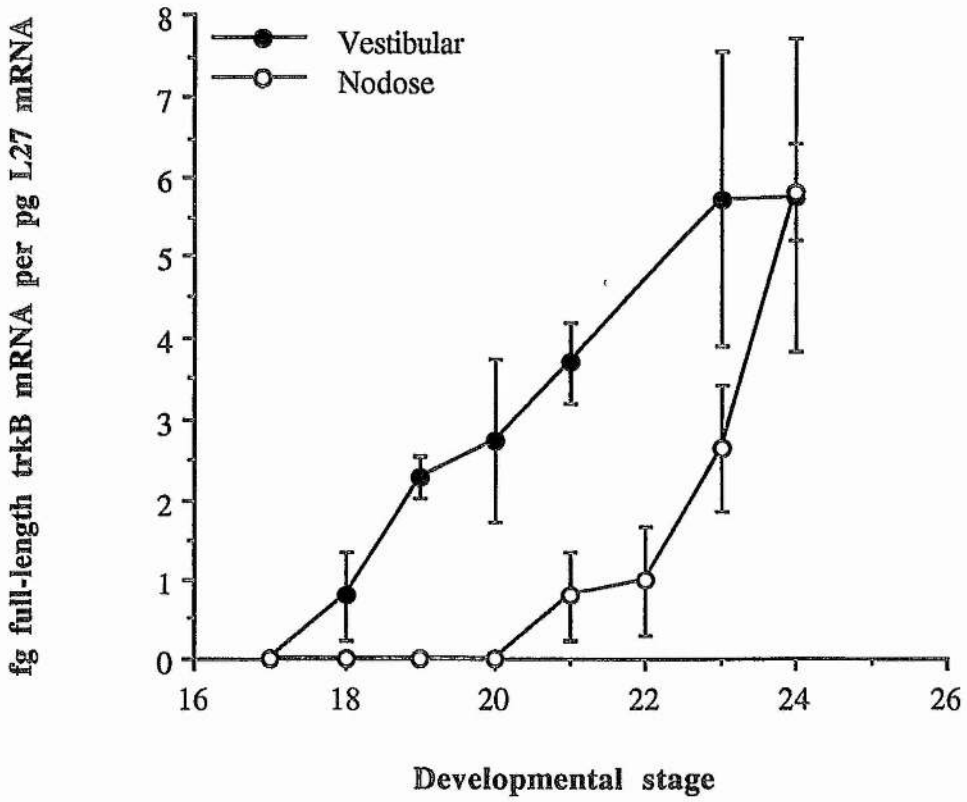


Figure 13 Graph of the level of full-length *trk*B mRNA relative to L27 mRNA (fg *trk*B mRNA per pg L27 mRNA) in the vestibular and nodose ganglia of chicken embryos at stages 17 to 24. The means and standard errors of measurements from 2 to 4 separate sets of ganglia are shown.

ganglia during the early stages of their development to see if the expression of this transcript closely follows that of *trk*B mRNA, as is the case for p75 mRNA and *trk*A mRNA expression in developing trigeminal ganglia (Wyatt and Davies, 1993). The level of p75 mRNA was measured in total RNA extracted from staged vestibular and nodose ganglia by quantitative, competitive RT-PCR and is expressed relative to L27 mRNA (figure 14). In both vestibular and nodose ganglia, p75 mRNA was clearly detected before full-length *trk*B mRNA (as early as stage 17 in vestibular ganglia and stage 20 in nodose ganglia, the earliest stages studied in each case). There were marked differences in the relative levels of p75 mRNA in the vestibular and nodose ganglia. The level of p75 mRNA remained low in the vestibular ganglion from stages 20 to 24, while the level of p75 mRNA increased 3-fold in the nodose ganglion between stages 20 and 24. At stage 24, the level of p75 mRNA was 9-fold greater in the nodose ganglion compared to the vestibular ganglion, while at the same stage of development, the levels of *trk*B mRNA were similar in both of these ganglia (figure 13).

Expression of BDNF mRNA in the Peripheral and Central Targets of Vestibular and Nodose Neurons

To determine whether BDNF mRNA is expressed in the central or peripheral targets of the vestibular and nodose ganglia and to determine the relationship between expression and the timing of innervation, quantitative RT-PCR was used to measure the level of BDNF mRNA in the hindbrain (the common central target field of the vestibular and nodose ganglia), otic vesicle (innervated by the vestibulocochlear neurons), and heart (one of the peripheral innervation targets of the nodose ganglion). Figure 15 shows the level of BDNF mRNA in these tissues relative to L27 mRNA from stages 16 to 24. BDNF mRNA was detected in all three tissues throughout this period of development. Although the level of BDNF mRNA relative to L27 mRNA was highest in the otic vesicle and lowest in heart, there was little change in the overall level of BDNF mRNA in each tissue between stages 16 to 24. This indicates that BDNF mRNA is expressed in these tissues prior to the arrival of the earliest sensory axons

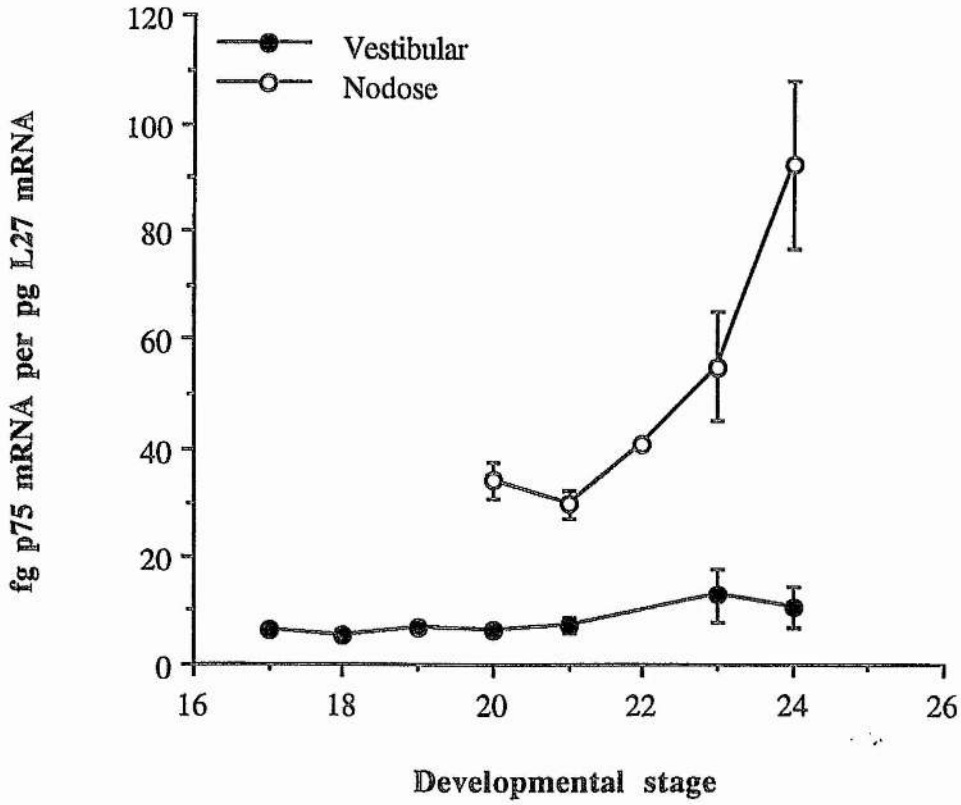


Figure 14 Graph of the level of p75 mRNA relative to L27 mRNA (fg p75 mRNA per pg L27 mRNA) in the vestibular and nodose ganglia of chicken embryos at stages 17 to 24. The means and standard errors from 2 to 4 separate sets of ganglia at each stage are shown (stage 20 was was the earliest stage at which the nodose ganglia were studied).

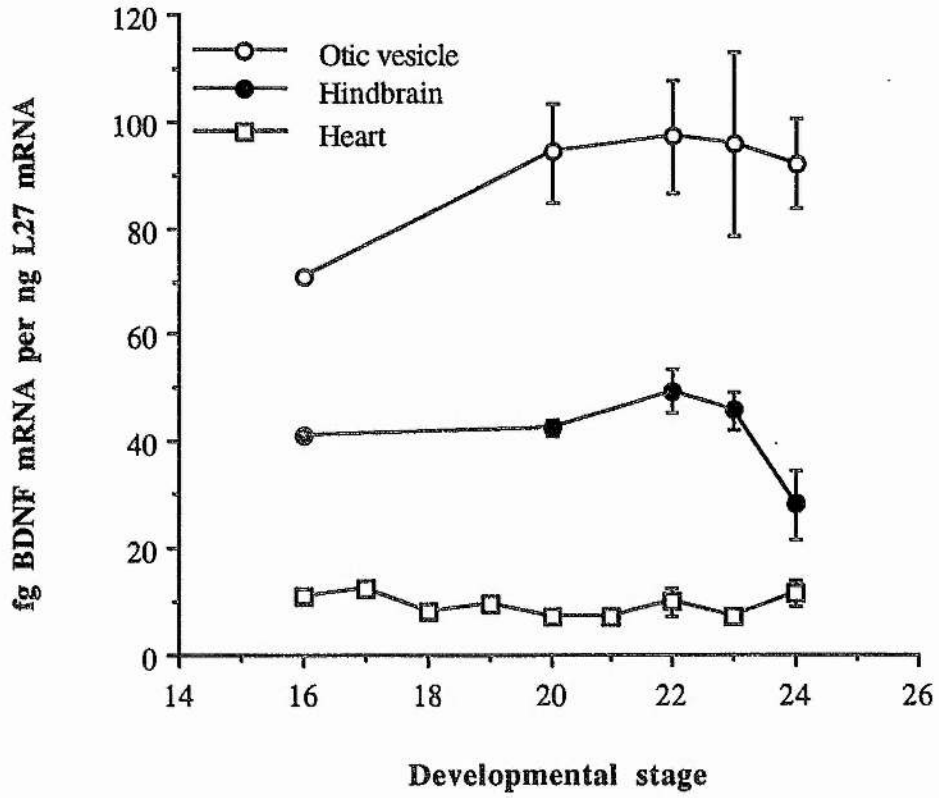


Figure 15 Graph of the level of BDNF mRNA relative to L27 mRNA (fg BDNF mRNA per ng L27 mRNA) in the heart, hindbrain, and otic vesicle of chicken embryos at stages 16 to 24. The means and standard errors of measurements from 1 (stage 16 only) to 3 separate sets of hindbrains and otic vesicles and 2 to 4 separate sets of hearts at each stage are shown.

and that there is no obvious increase in BDNF mRNA concentration during the early stages of target field innervation.

Regulation of *trk*B mRNA Expression in Cultured Nodose Neurons

To investigate the regulation of *trk*B mRNA expression during the early stages of sensory neuron development, the levels of this transcript were measured in very low density cultures of nodose ganglion neurons. These neurons were placed in culture at stage 19 before they have innervated their targets and become dependent on BDNF for survival (Vogel and Davies, 1991). Figure 16 shows the level of *trk*B mRNA in these neurons grown with and without BDNF in the culture medium. *trk*B mRNA was not detectable in these cultures until after 36 hours incubation. At this time and at 48 hours there was no significant difference between the level of *trk*B mRNA in control cultures and BDNF-supplemented cultures. There were significantly higher levels of *trk*B mRNA in BDNF-supplemented cultures after 60 hours and by 72 hours the level of *trk*B mRNA was approximately 3-fold higher in BDNF-supplemented cultures.

Discussion

The present studies of the timing and regulation of *trk*B mRNA expression have clarified how BDNF responsiveness is controlled in developing sensory neurons and provided interesting contrasts with previous developmental studies of the expression of *trk*A mRNA which codes for the NGF tyrosine kinase receptor. *trk*B mRNA was expressed in the developing vestibular ganglion before the nodose ganglion. This accords with the earlier response of vestibular neurons to BDNF *in vitro* compared with nodose neurons (Vogel and Davies, 1991), suggesting that the timing of BDNF responsiveness is controlled by the expression of its receptor. In contrast, Wyatt and Davies (1993, 1995) have shown that *trk*A mRNA is expressed at low levels in developing sensory and sympathetic neurons before these neurons show any obvious response to NGF, although there is a marked increase in the expression of *trk*A mRNA with the acquisition of NGF responsiveness. Although *trk*B mRNA has previously

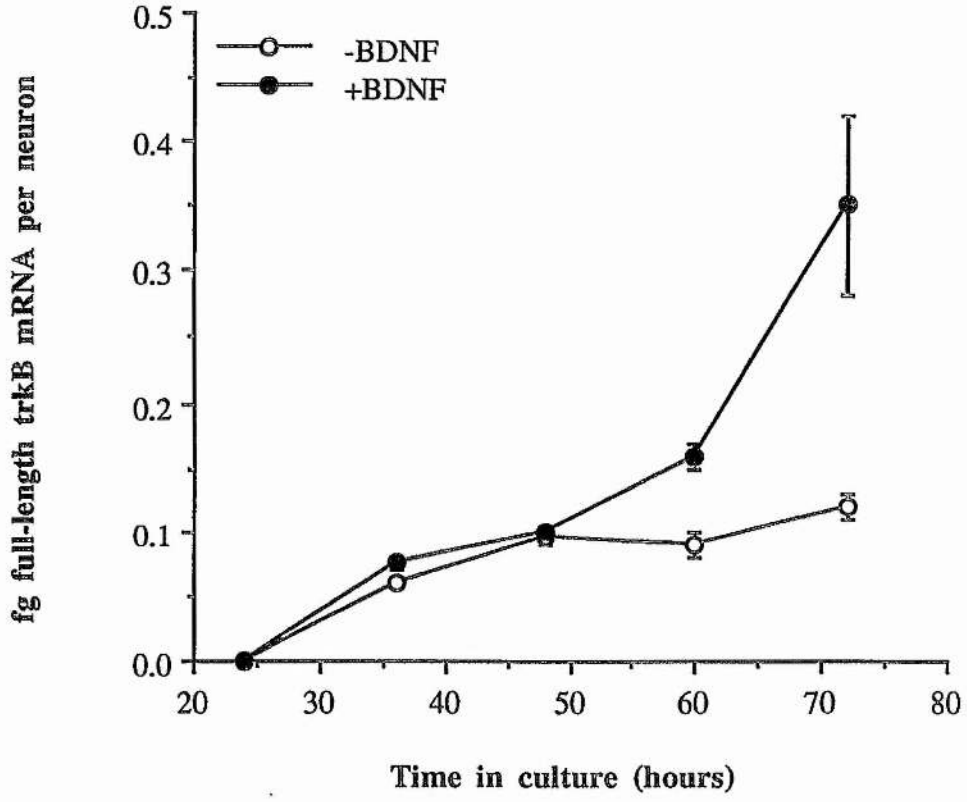


Figure 16 Graph of the level of full-length *trk*B mRNA (fg per neuron) at 12 hourly intervals in low-density cultures of stage 19 chicken nodose neurons grown without or with BDNF in the culture medium for 72 hours. The means and standard errors of measurements from triplicate dishes of both experimental conditions at each time point are shown.

been detected in the vestibular and nodose ganglia of the rat embryo by *in situ* hybridization, the relative timing of *trk*B mRNA expression in these two ganglia was not studied (Ernfors *et al.*, 1992).

It was shown in the present study that nodose neurons cultured early during the phase of neurotrophin independent survival (stage 19) start expressing *trk*B mRNA after 36 hours *in vitro*. Although BDNF does not enhance the survival of these neurons until after 72 hours *in vitro* (Vogel and Davies, 1991), BDNF exposure between 48 and 72 hours does give rise to two other effects. It induces an increase in cell body size and accelerates the acquisition of BDNF-dependent survival as suggested by the observation that neurons die more rapidly and extensively after 72 hours following BDNF deprivation compared with neurons that have not been exposed to BDNF *in vitro* (Vogel and Davies, 1991). Interestingly, the accelerated acquisition of BDNF dependence is brought about by exposure to a 12- or 24-hour pulse of BDNF only between 48 and 72 hours; pulses of BDNF administered prior to 48 hours *in vitro* do not accelerate BDNF dependence. In the present study, BDNF was shown to increase the expression of *trk*B mRNA between 48 and 72 hours *in vitro* but failed to influence either the onset of *trk*B mRNA expression or level of *trk*B mRNA for the first 48 hours *in vitro*. The temporal coincidence of the upregulation of *trk*B mRNA expression and acceleration of BDNF dependence by BDNF raises the possibility that the enhanced BDNF dependence of BDNF-exposed neurons is due to an increase in the expression of BDNF receptors.

The effects of BDNF on *trk*B mRNA expression observed in this study differ from those of NGF on *trk*A mRNA expression in developing sensory neurons. While BDNF was shown to increase the expression of *trk*B mRNA in cultured nodose ganglion neurons shortly before they become BDNF-dependent, Wyatt and Davies (1993) have found that NGF does not affect *trk*A mRNA expression in cultured mouse trigeminal neurons at either the equivalent stage or later stages of development. They have also found that *trk*A mRNA expression increases normally in the early trigeminal ganglia of mouse embryos that are homozygous for a null mutation in the NGF gene,

indicating that *trk*A expression is regulated independently of NGF in developing sensory neurons. Likewise, *trk*A mRNA is regulated independently of NGF in developing sympathetic neurons *in vitro* (Wyatt and Davies, 1995).

Studies using antisense BDNF oligonucleotides to inhibit BDNF synthesis in cultured embryonic chicken DRG neurons have shown that BDNF acts by an autocrine loop to promote an early maturational change in many of these neurons before they become dependent on added neurotrophins for survival (Wright *et al.*, 1992). Similar *in vitro* experiments on adult DRG neurons have also shown that a BDNF autocrine loop operates in many of these neurons to maintain survival (Acheson *et al.*, 1995). It was demonstrated in the present study that nodose neurons do not express *trk*B mRNA during the first 24 hours of the phase of neurotrophin independence, clearly indicating that BDNF cannot act by an autocrine route via its tyrosine kinase receptor during this early period of development. Thus, a BDNF autocrine loop is not a characteristic of all newly differentiated sensory neurons and is not required for their early survival in the absence of exogenous neurotrophins. The demonstration that reduction of intracellular free-Ca^{2+} or depletion of intracellular Ca^{2+} stores kills early neurotrophin-independent nodose neurons but has negligible effect on older neurons growing in the presence of BDNF suggests that intracellular Ca^{2+} plays an important role in regulating neuronal survival during this stage of development (Larmet *et al.*, 1992).

The developmental changes in the level of p75 mRNA expression relative to *trk*B mRNA in vestibular and nodose ganglia differ from the findings of previous quantitative studies of changes in the level of p75 mRNA expression relative to *trk*A mRNA in ganglia of NGF-dependent neurons. Wyatt and Davies (1993, 1995) have shown that in both trigeminal ganglia and superior cervical sympathetic ganglia (SCG), p75 and *trk*A mRNAs are co-expressed from the earliest stages, while in the present study, p75 mRNA expression was found to precede that of *trk*B mRNA in developing vestibular and nodose ganglia. The demonstration by *in situ* hybridization that p75 mRNA is expressed in the epibranchial placode that gives rise to nodose neurons (Hallböök *et al.*, 1990) suggests that p75 may be expressed prior to neuronal

differentiation by the sensory neuron progenitor cells of this ganglion. The levels of p75 mRNA and *trk*B mRNA were found to increase to a similar extent from stages 21 to 24 in the nodose ganglion, while the level of p75 mRNA was found to be much lower than that of *trk*B mRNA in the vestibular ganglion and showed no increase over the equivalent period of development (compare figures 13 and 14). The relationship between p75 and *trk*B mRNA expression in the developing nodose ganglion is similar to the relationship between p75 and *trk*A mRNA expression in the developing trigeminal ganglion where the levels of these mRNAs increase in parallel (Wyatt and Davies, 1993). The relationship between p75 and *trk*B mRNA expression in the developing vestibular ganglion is similar to the relationship between p75 and *trk*A mRNA expression in the developing SCG where the level of p75 mRNA remains low in the early stages of development of this ganglion (Wyatt and Davies, 1995). In the present study, it was not ascertained whether the level of p75 mRNA in vestibular ganglia increases later in development (as it does in SCG). However, *in situ* hybridization studies of p75 mRNA expression in chicken embryos have shown that the grain density is much higher over the nodose ganglion compared with the vestibular ganglion at stage 27 (Hallböök *et al.*, 1990), suggesting that p75 mRNA continues to be expressed at a low level in the vestibular ganglion later in development. While the differing patterns of *trk*A and p75 expression in trigeminal and SCG neurons modulate the sensitivity of these neurons to NGF at different stages of development (Wyatt and Davies, 1993, 1995), the functional significance of the differing patterns of *trk*B and p75 expression in the BDNF-dependent vestibular and nodose neurons is unclear.

Interpretation of the relative differences in the levels of transcripts for p75 and *trk* tyrosine kinase receptors in whole ganglia is potentially complicated by the presence of both neurons and non-neuronal cells in these ganglia. Transcripts for the catalytic *trk*B receptor have been detected in various neurons and not in non-neuronal cells (Klein *et al.*, 1990b; Merlio *et al.*,1992; Beck *et al.*, 1993; Frisen *et al.*,1993; Funakoshi *et al.*, 1993; Rudge *et al.*, 1994; Biffo *et al.*, 1995), while p75 mRNA and p75 protein are expressed in both neurons and a variety of non-neuronal cells (Ernfors

et al., 1988; Scarpini *et al.*, 1988; Yan and Johnson, 1988; Hallböök *et al.*, 1990; Wyatt *et al.*, 1990; von Bartheld *et al.*, 1991; Wheeler and Bothwell, 1992). However, in late embryonic trigeminal and SCG, when neurons become clearly distinguishable morphologically and can be separated from the non-neuronal cells, p75 mRNA is detectable only in the neurons (Wyatt and Davies, 1993, 1995). Thus, regardless of the presence of non-neuronal cells in developing ganglia, the overall levels of transcripts for p75, *trk*A and catalytic *trk*B in whole ganglia are likely to reflect the relative levels of these transcripts in neurons.

In the present study, BDNF mRNA was found to be expressed in both the central and peripheral target fields of the vestibular and nodose ganglia from as early as stage 16, with very little change in the level of BDNF mRNA relative to L27 mRNA throughout the period most vestibular and nodose axons reach these targets. Axon tracing studies have shown that vestibular axons start entering the hindbrain between stages 16 and 17 and that the earliest nodose axons enter the hindbrain at stage 19. The peripheral axons of vestibular neurons are present in the otic vesicle as early as stage 21 and the peripheral axons of the nodose ganglion do not reach the heart until between stages 24 and 26 (Vogel and Davies, 1991). Thus, it is likely that BDNF synthesis begins in these tissues prior to the arrival of the earliest sensory axons. Likewise, BDNF mRNA has been detected in the peripheral target field of the mouse trigeminal ganglion at least a day before the arrival of the earliest sensory axons (Buchman and Davies, 1993), whereas NGF protein and message are first detected with the arrival of the earliest sensory axons (Davies *et al.*, 1987). Although the present study has focused on the timing of BDNF mRNA expression, *in situ* hybridization studies of the E4 (stage 24) chicken embryo have localized BDNF mRNA to the regions of the otic vesicle epithelium that give rise to the vestibular apparatus (Hallböök *et al.*, 1993). *In situ* hybridization has also localized BDNF mRNA to regions of the early otic vesicle of rat embryos that give rise to both vestibular and cochlear sensory structures (Pirvola *et al.*, 1992).

References

Acheson, A., Conover, J.C., Fandl, J.P., DeChlara, T.M., Russell, M., Thadani, A., Squinto, S.P., Yancopoulos, G.D., Lindsay, R.M. (1995). A BDNF autocrine loop in adult sensory neurons prevents cell death. *Nature* **374**: 450-453.

Adler, R., Landa, K.B., Manthorpe, M., Varon, S. (1979). Cholinergic neurononeurotrophic factors: intraocular distribution of soluble trophic activity for ciliary neurons. *Science* **204**: 1434-1436.

Allsopp, T.E., Robinson, M., Wyatt, S. Davies, A.M. (1993). Ectopic *trk*A expression mediates a NGF survival response in NGF-independent sensory neurons but not in parasympathetic neurons. *J. Cell Biol.* **123**: 1555-1566.

Aloe, L., Alleva, E., Bohm, A., Levi-Montalcini, R. (1986). Aggressive behaviour induces release of nerve growth factor from mouse salivary gland into the bloodstream. *Proc. Natl Acad. Sci. USA* **83**: 6184-6187.

Aloe, L., Cozzari, C., Calissano, P., Levi-Montalcini, R. (1981). Somatic and behavioural postnatal effects of fetal injections of nerve growth factor antibodies in the rat. *Nature* **291**: 413-415.

Angeletti, R.H., Bradshaw, R.A. (1971). Nerve growth factor from mouse submaxillary gland: Amino acid sequence. *Proc. Natl Acad. Sci. USA* **68**: 2417-2420.

Angeletti, R.H., Hermodson, M.A., Bradshaw, R.A. (1973). Amino acid sequences of mouse 2.5S nerve growth factor. II. Isolation and characterization of the thymolytic and peptic peptides and the complete covalent structure. *Biochemistry* **12**: 100-115.

Arakawa, Y., Sendtner, M., Thoenen, H. (1990). Survival effect of ciliary neurotrophic factor (CNTF) on chick embryonic motoneurons in culture: comparison with other neurotrophic factors and cytokines. *J. Neurosci.* **10**: 3507-3515.

Ayer-LeLievre, C., Olson, L., Ebendal, T., Seiger, Å. Persson, H. (1988). Expression of the β-nerve growth factor in hippocampal neurons. *Science* **240**:

1339-1341.

Bailey, G.S., Banks, B.E.L., Peirce, F.L., Shipolini, R.A. (1975). A comparative study of nerve growth factors from snake venoms. *Comp. Biochem. Physiol.* **51B**: 429-438.

Baimbridge, K.G., Celio, M.R., Rogers, J.H. (1992). Calcium-binding proteins in the nervous system. *Trends Neurosci.* **15**: 303-308.

Barbacid, M. (1993). Nerve growth factor: a tale of two receptors. *Oncogene* **8**: 2033-2042.

Barbacid, M., Lamballe, F., Pulido, D., Klein, R. (1991). The *trk* family of tyrosine protein kinase receptors. *Biochemica et Biophysica Acta* **1072**: 115-152.

Barbin G., Manthorpe, M., Varon, S. (1984). Purification of the chick eye ciliary neurotrophic factor. *J. Neurochem.* **43**: 1468-1478.

Barde, Y-A. (1989). Trophic factors and neuronal survival. *Neuron* **2**: 1525-1534.

Barde, Y-A., Edgar, D., Theonen, H. (1982). Purification of a new neurotrophic factor from mammalian brain. *EMBO J.* **1**: 549-553.

Barker, P.A., Lomen-Hoerth, C., Gensch, E.M., Meakin, S.O., Glass, D.J., Shooter, E.M. (1993). Tissue-specific alternative splicing generates two isoforms of the *trk*A receptor. *J. Biol. Chem.* **268**: 15150-15157.

Barker, P.A., Murphy, R.A. (1992). The nerve growth factor receptor: a multicomponent system that mediates the actions of the neurotrophin family of proteins. *Mol. Cell. Biochem.* **110**: 1-15.

Battleman, D.S., Geller, A.I., Chao, M.V. (1993). HSV-1 mediated gene transfer of the human nerve growth factor receptor $p75^{hNGFR}$ defines high affinity NGF binding. *J. Neurosci.* **13**: 941-951.

Beck, K.D., Lamballe, F., Klein, R., Barbacid, M., Schauwecker, P.E., McNeill, T.H., Finch, C.E., Hefti, F., Day, J.R. (1993). Induction of noncatalytic *trk*B neurotrophin receptors during axonal sprouting in the adult hippocampus. *J. Neurosci.* **13**: 4001-4014.

Beck, K.D., Valverde, J., Alexi, T., Poulsen, K., Moffat, B., Vandlen, R.A.,

Rosenthal, A., Hefti, F. (1995). Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. *Nature* **373**: 339-341.

Berg, M.M, Sternberg, D.W., Hempstead, B.L., Chao, M.V. (1990). The low-affinity p75 nerve growth factor (NGF) receptor mediates NGF-induced tyrosine phosphorylation. *Proc. Natl Acad. Sci. USA* **88**: 7106-7110.

Berkemeier, L.R., Winslow, J.W., Kaplan, D.R., Nikolics, K., Goeddel, D.V., Rosenthal, A. (1991). Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. *Neuron* **7**: 857-866.

Biffo, S., Offenhauser, N., Carter, B.D., Barde, Y-A. (1995). Selective binding and internalization by truncated receptors restrict the availability of BDNF during development. *Development* **121**: 2461-2470.

Birren, S.J., Anderson, D.J. (1990). A v-myc immortalized sympathoadrenal progenitor cell line in which neuronal differentiation is initiated by FGF but not NGF. *Neuron* **4**: 189-201.

Birren, S.J., Lo, L., Anderson, D.J. (1993). Sympathetic neurons undergo a developmental switch in trophic dependence. *Development* **119**: 590-610.

Birren, S.J., Verdi, J.M., Anderson, D.J. (1992). Membrane depolarization induces $p140^{trk}$ and NGF responsiveness, but not $p75^{LNGFR}$, in MAH cells. *Science* **257**: 395-397.

Brand, N.J., Petkovich, M.,Krust, A., Chambon, P., de Thé, H., Marchio, A., Tiollais, P., Dejean, A. (1988). Identification of a second human retinoic acid receptor. *Nature* **332**: 850-853.

Buchman, V.L., Davies, A.M. (1993). Different neurotrophins are expressed and act in a developmental sequence to promote the development of embryonic sensory neurons. *Development* **118**: 989-1001.

Buchman, V.L., Sporn, M., Davies, A.M. (1994). Role of transforming growth factor-beta isoforms in regulating the expression of nerve growth factor and neurotrophin-3 mRNA levels in embryonic cutaneous cells at different stages of

development. *Development* **120**: 1621-1629.

Buj-Bello, A.M., Pinon, L., Davies, A.M. (1994). The survival of NGF-dependent but not BDNF-dependent cranial sensory neurons is promoted by several different neurotrophins early in their development. *Development* **120**: 1573-1580.

Cantley, L.C., Auger, K., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., Soltoff, S. (1991). Oncogenes and signal transduction. *Cell* **64**: 281-304.

Carnahan, J.F., Patel, D.R., Miller, J.A. (1994). Stem cell factor is a neurotrophic factor for neural crest-derived chick sensory neurons. *J. Neurosci.* **14**: 1433-1440.

Cattaneo, E., McKay, R. (1990). Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. *Nature* **347**: 762-765.

Chalazonitis, A., Kalberg, J., Twardzik, D.R., Morrison, R.S., Kessler, J.A. (1992). Transforming growth factor β has neurotrophic effects on sensory neurons *in vitro* an is synergistic with nerve growth factor. *Dev. Biol.* **152**: 121-132.

Chao, M.V. (1992). Neurotrophin receptors: a window into neuronal differentiation. *Neuron* **9**: 583-593.

Chao, M.V. (1994). The p75 neurotrophin receptor. *J. Neurobiol.* **25**: 1373-1385.

Chao, M.V., Bothwell, M.A., Ross, A., Koprowski, H., Lanahan, A.A., Buck, C.R., Sehgal, A. (1986). Gene transfer and molecular cloning of the human NGF receptor. *Science* **232**: 518-521.

Chapman, C.A., Banks, B.E.C., Vernon, C.A., Walker, J.M. (1981). The isolation and characterization of nerve growth factor from the prostate gland of the guinea-pig. *Eur. J. Biochem.* **115**: 347-351.

Chomoczynski, P., Saachi, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**: 156-159.

Chun, L.L., Patterson, P.H. (1977). The role of NGF in the development of rat sympathetic neurons *in vitro*. II Developmental studies. *J. Cell Biol.* **75**: 705-

711.

Claude, P., Parada, I.M., Gordon, K.A., D'Amore, P.A., Wagner, J.A. (1988). Acidic fibroblast growth factor stimulates adrenal chromaffin cells to proliferate and to extend neurites, but is not a long-term survival factor. *Neuron* **1**: 783-790.

Cohen, S. (1960). Purification and metabolic effects of a nerve growth-promoting protein from the mouse salivary gland and its neuro-cytotoxic antiserum. *Proc. Natl Acad. USA* **46**: 302-311.

Cohen, S., Levi-Montalcini, R., Hamburger, V. (1954). A nerve growth stimulating factor isolated from sarcoma 37 and 180. *Proc. Natl Acad. Sci. USA* **40**: 1014-1018.

Collins, F. Lile, J.D. (1989). The role of dihydropyridine-sensitive voltage gated calcium channels in potassium mediated neuronal survival. *Brain Res.* **502**: 99-108.

Collins, F., Schmidt, M.F., Guthrie, P.B., Kater, S.B. (1991). Sustained increase in intracellular calcium promotes neuronal survival. *J. Neurosci.* **11**: 2582-2587.

Cordon-Cardo, C., Tapley, P., Jing, S., Nanduri, V., O'Rourke, E., Lamballe, F., Kovary, K., Klein, R., Jones, K.R., Reichardt, L.F., Barbacid, M. (1991). The *trk* tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. *Cell* **66**: 173-183.

Coughlin, M.D., Collins, M.B. (1985). Nerve growth factor-independent development of embryonic mouse sympathetic neurons in dissociated cell culture. *Dev. Biol.* **110**: 392-401.

Cowan, W.M., Fawcett, J.W., O'Leary, D.D.M., Stanfield, B.B. (1984). Regressive events in neurogenesis. *Science* **225**: 1258-1265.

Crowley, C., Spencer, S.D., Nishimura, M.C., Chen, K.S., Pitts-Meek, S., Armanini, M.P., Ling, L.H., McMahon, S.B., Shelton, D.L., Levinson, A.D., Phillis, H.S. (1994). Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic

neurons. *Cell* **76**: 1001-1011.

D'Amico, M.A. (1982). Temporal patterns of neurogenesis in avian cranial sensory and autonomic ganglia. *Am. J. Anat.* **163**: 351-372.

D'Amico, M.A., Noden, D.M. (1980). An autoradiographic analysis of the development of the chick trigeminal ganglion. *J. Embryol. Exp. Morphol.* **55**: 167-182.

D'Amico, M.A., Noden, D.M. (1983). Contributions of placodal and neural crest cells to avian cranial peripheral ganglia. *Am. J. Anat.* **166**: 445-468.

Davies, A.M. (1986). The survival and growth of embryonic proprioceptive neurons is promoted by a factor present in skeletal muscle. *Dev. Biol.* **115**: 56-67.

Davies, A.M. (1987). Molecular and cellular aspects of patterning sensory neuron connections in the vertebrate nervous system. *Development* **101**: 185-208.

Davies, A.M. (1988a). Role of neurotrophic factors in development. *Trends Genetics* **4**: 139-143.

Davies, A.M. (1988b). Neurotrophic factor bioassay using dissociated neurons. *Nerve Growth Factors* (ed. R.A. Rush; pub. John Wiley & Sons Ltd.), p. 96-105.

Davies, A.M. (1989). Intrinsic differences in the growth rate of early nerve fibres related to target distance. *Nature* **337**: 553-555.

Davies, A.M. (1992). Cell death and the trophic requirements of developing sensory neurons. In *Sensory Neurons: Diversity, Development, and Plasticity*, S. Scott, ed. (Oxford University Press) p.194-214.

Davies, A.M. (1994a) The role of neurotrophins during successive stages of sensory neuron development. *Prog. Growth Factor Res.* **5**: 263-289.

Davies, A.M. (1994b). Intrinsic programmes of growth and survival in developing vertebrate neurons. *Trends Neurosci.* **17**: 195-199.

Davies, A.M. (1994c) The role of neurotrophins in the developing nervous system. *J. Neurobiol.* **25**: 1334-1348.

Davies, A.M., Bandtlow, C., Heumann, R., Korsching, S., Rohrer, H., Thoenen, H. (1987). Timing and site of nerve growth factor synthesis in developing skin in

relation to innervation and expression of the receptor. *Nature* **326**: 353-358.

Davies, A.M., Horton, A., Burton, L.E., Schmelzer, C., Vandlen, R., Rosenthal, A. (1993a). Neurotrophin-4/5 is a mammalian-specific survival factor for distinct populations of sensory neurons. *J. Neurosci.* **13**: 4961-4967.

Davies, A., Lee, K-F., Jaenisch, R. (1993b). p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. *Neuron* **1**: 565-575.

Davies, A.M., Lindsay, R.M. (1985). The avian cranial sensory ganglia in culture: Differences in the response of placode-derived and neural crest-derived neurons to nerve growth factor. *Dev. Biol.* **111**: 62-72.

Davies, A.M., Lumsden, A.G.S. (1984). Relation of target encounter and neuronal death to nerve growth factor responsiveness in the developing mouse trigeminal ganglion. *J. Comp. Neurol.* **223**: 124-137.

Davies, A.M., Lumsden, A.G., Rohrer, H. (1987). Neural crest-derived proprioceptive neurons express nerve growth factor receptors but are not supported by nerve growth factor in culture. *Neuroscience* **20**: 37-46.

Davies, A.M., Lumsden, A.G.S., Slavkin, H.C., Burnstock, G. (1981). Influence of nerve growth factor on the embryonic mouse trigeminal ganglion in culture. *Dev. Neurosci.* **4**: 150-156.

Davies, A.M., Thoenen, H., Barde, Y-A. (1986a) The response of chick sensory neurons to brain-derived neurotrophic factor. *J. Neurosci.* **6**: 1897-1904.

Davies, A.M., Thoenen, H., Barde, Y-A. (1986b). Different factors from the central nervous system and periphery regulate the survival of sensory neurons. *Nature* **319**: 497-499.

Davies, A.M., Wright, E.M. (1995). Neurotrophin autocrine loops. *Curr. Biol.* **5**: 723-726.

Davis, B.M., Lewin, G.R., Mendell, L.M., Jones, M.E., Albers, K.M. (1993). Altered expression of nerve growth factor in the skin of transgenic mice leads to changes in response to mechanical stimuli. *Neuroscience* **56**: 789-792.

Davis, S., Yancopoulos, G.D. (1993). The molecular biology of the CNTF receptor. *Curr. Opin. Neurobiol.* 3: 20-24.

Déchant, G., Biffo, S., Okazawa, H., Kolbeck, R., Pottgiesser, J., Barde, Y-A. (1993a). Expression and binding characteristics of the BDNF receptor chick *trk*B. *Development* **119**: 545-558.

Déchant, G., Rodriguez, T.A., Kolbeck, R., Barde, Y-A. (1993b). Specific high-affinity receptors for neurotrophin-3 on sympathetic neurons. *J. Neurosci.* 3: 2610-2616.

DiCocco-Bloom, E., Black, I.B. (1988). Insulin growth factors regulate the mitotic cycle in cultured rat sympathetic neuroblasts. *Proc. Natl. Acad. Sci. USA* **85**: 4066-4070.

DiCocco-Bloom, E. Friedman, W.J., Black, I.B. (1993). NT-3 stimulates sympathetic neuroblast proliferation by promoting precursor cell survival. *Neuron* **11**: 1101-1111.

Doherty, P., Seaton, P., Flanigan, T.P., Walsh, F.S. 1988. Factors controlling the expression of the NGF receptor in PC12 cells. *Neurosci. Lett.* **92**: 222-227.

Ebendal, T. (1992). Function and evolution in the NGF family and its receptors. *J. Neurosci. Res.* **32**: 461-470.

Edwards, R.H., Selby, M.J., Garcia, P.D., Rutter, W.J. (1988). Processing of the native nerve growth factor to form biologically active nerve growth factor. *J. Biol. Chem.* **263**: 6810-6815.

Ernfors, P., Hallböök. F., Ebendal, T., Shooter, E.M., Radeke, M.J., Misko, T.P., Persson, H. (1988). Developmental and regional expression of beta-nerve growth factor receptor mRNA in the chick and rat. *Neuron* 1: 983-986.

Ernfors, P., Henschen, A., Olsen, L., Persson, H. (1989). Expression of nerve growth factor receptor mRNA is developmentally regulated and increased after axotomy in rat spinal cord motoneurons. *Neuron* 2: 1605-1613.

Ernfors, P., Ibáñez, C.F., Ebendal, T., Olson, L., Persson, H. (1990a). Molecular cloning and neurotrophic activities of a protein with structural similarities to

nerve growth factor: developmental and topographical expression in the brain. *Proc. Natl. Acad. Sci. USA* **87**: 5454-5458.

Ernfors, P., Lee, K-F., Jaenisch, R., (1994a). Mice lacking brain-derived neurotrophic factor develop sensory deficits. *Nature* **368**: 147-150.

Ernfors, P., Lee, K-F., Kucera, J., Jaenisch, R. (1994b). Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. *Cell* **77**: 503-512.

Ernfors, P., Merlio, J., Persson, H. (1992). Cells expressing mRNA for neurotrophins and their receptors during embryonic rat development. *Euro. J. Neurosci.* **4**: 1140-1158.

Ernfors, P., Persson, H. (1991). Developmentally regulated expression of HDNF/NT-3 mRNA in rat spinal cord motoneurons and expression of BDNF mRNA in embryonic dorsal root ganglion. *Eur. J. Neurosci.* **3**: 953-961.

Ernfors, P., Wetmore, C, Olson, L., Persson, H. (1990b). Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. *Neuron* **5**: 511-526.

Ernsberger, U., Edgar, D., Rohrer, H. (1989a). The survival of early chick sympathetic neurons *in vitro* is dependent on a suitable substrate but independent of NGF. *Dev. Biol.* **135**: 250-262.

Ernsberger, U., Rohrer, H. (1988). Neuronal precursor cells in chick dorsal root ganglia: differentiation and survival *in vitro*. *Dev. Biol.* **126**: 420-432.

Ernsberger, U., Sendtner, M., Rohrer, H. (1989b). Proliferation and differentiation of embryonic chick sympathetic neurons: effects of ciliary neurotrophic factor. *Neuron* **2**: 1275-1285.

Esch, F., Baird, A., Ling, N., Ueno, N., Hill., F., Denoroy, L., Klepper, R., Gospodarowicz, D., Bhlen, P., Guillemin, R. (1985). Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-terminal sequence of bovine acidic FGF. *Proc. Natl Acad. Sci.* **82**: 6507-6511.

Folkman, J., Klagsbrun, M. (1987). Angiogenic factors. *Science (Wash . DC)* **235**: 442-447.

Franklin, J.L., Johnson, Jr., E.M. (1992). Suppression of programmed neuronal death by sustained elevation of cytoplasmic calcium. *Trends Neurosci.* **15**: 501-508.

Frisen, J., Verge, V.M., Fried, K., Risling, M., Persson, H., Trotter, J., Hökfelt, T., Lindholm, D. (1993). Characterization of glial *trk*B receptors: differential response to injury in the central and peripheral nervous systems. *Proc. Natl. Acad. Sci. USA* **90**: 4971-4975.

Funakoshi, H., Frisen, J., Barbany, G., Timmusk, T., Zachrisson, O., Verge, V.M., Persson, H. (1993). Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. *J. Cell Biol.* **123**: 455-465.

Gaese, F., Kolbeck, R., Barde, Y-A. (1994). Sensory ganglia require neurotrophin-3 early in development. *Development* **120**: 1613-1619.

Gall, C.M., Isackson, P.J. (1989). Limbic seizures increase neuronal production of messenger RNA for nerve growth factor. *Science* **245**: 758-761.

Giguère, V., Ong, E.S., Segui, P., Evans, R.M. (1987). Identification of a receptor for the morphogen retinoic acid. *Nature* **330**: 624-629.

Glass, D.J., Nye, S.H., Hantzopoulos, P., Macchi, M.J., Squinto, S.P., Goldfarb, M., Yancopoulos, G.D. (1991). *trk*B mediates BDNF-NT-3 dependent survival and proliferation in fibroblasts lacking the low-affinity NGF receptor. *Cell* **66**: 405-413.

Greene, L.A. (1977). Quantitative *in vitro* studies on the nerve growth factor (NGF) requirement of neurons. I Sympathetic neurons. *Dev. Biol.* **58**: 96-105.

Greene, L.A. and Shooter, E.M. (1980). The nerve growth factor: biochemistry, synthesis and mechanism of action. *Ann. Rev. Neurosci.* **3**: 353-402.

Gospodarowicz, D., Ferrara, N., Schweigerer, L., Neufeld, G. (1987). Structural characterization and biological functions of fibroblast growth factor. *Endocr. Rev.* **8**: 95-114.

Götz, R., Koster, R., Winkler, K., Raulf, F., Lottspeich, F., Schartl, M., Thoenen, H. (1994). Neurotrophin-6 is a new member of the nerve growth factor family. *Nature* **372**: 266-269.

Hagg, T., Quon, D., Higaki, J., Varon, S. (1992). Ciliary neurotrophic factor prevents neuronal degeneration and promotes low-affinity NGF receptor expression in the adult rat CNS. *Neuron* **8**: 145-158.

Hallböök, F., Ayer, L.C., Ebendal, T., Persson, H. (1990). Expression of nerve growth factor receptor mRNA during early development of the chicken embryo: emphasis on cranial ganglia. *Development* **108**: 693-704.

Hallböök, F., Ibáñez, C.F., Ebendal, T., Persson, H. (1993). Cellular localization of brain-derived neurotrophic factor and neurotrophin-3 mRNA expression in the early chicken embryo. *Eur. J. Neurosci.* **5**: 1-14.

Hallböök, F., Ibáñez, C.F., Persson, H. (1991). Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in *Xenopus* ovary. *Neuron* **6**: 845-858.

Hamburger, V., Brunso-Bechtold, J.K., Yip, J.W. (1981). Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. *J. Neurosci.* **1**: 60-71.

Hamburger, V., Hamilton, H.L. (1951). A series of normal stages in the development of the chicken embryo. *J. Morphol.* **88**: 49-92.

Harper, S., Davies, A.M. (1990). NGF mRNA expression in developing cutaneous epithelium is related to innervation density. *Development* **110**: 515-519.

Harper, G.P. Thoenen, H. (1981). Target cells: histological effects and mechanism of action of nerve growth factor and its antibodies. *Ann. Rev. Pharmacol. Toxicol.* **21**: 201-229.

Hartikka, J., Hefti, F. (1988a). Comparison of nerve growth factor's effects on development of septum, striatum, and nucleus basalis cholinergic neurons *in vitro*. *J. Neurosci. Res.* **21**: 352-364.

Hartikka, J., Hefti, F. (1988b). Development of septal cholinergic neurons in culture:

plating density and glial cells modulate effects of NGF on survival, fibre growth, and expression of transmitter-specific enzymes. *J. Neurosci.* **8**: 2967-2985.

Haskell, B.E., Stach R.W., Werbach-Perez, K., Perez-Polo, J.R. (1987). Effect of retinoic acid on nerve growth factor receptors. *Cell Tissue Res.* **247**: 67-73.

Hatanaka, H., Tsuki, H., Hihonmatsu, I. (1988). Developmental change in the nerve growth factor action from induction of choline acetyl transferase to promotion of cell survival of cultured basal forebrain cholinergic neurons from postnatal rats. *Devl. Brain Res.* **467**: 85-95.

Hefti, F. (1986). Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transections. *J. Neurosci.* **6**: 2155-2162.

Hempstead, B.L., Martin-Zanca, D., Kaplan, D.R., Parada, L.F., Chao, M.V. (1991). High-affinity binding requires co-expression of the *trk* proto-oncogene and the low affinity NGF receptor. *Nature* **350**: 678-683.

Hempstead, B.L., Martin-Zanca, D., Patil, N.,Thiel, B., Chao, M.V. (1990). Deletion of cytoplasmic sequences of the nerve growth factor receptor leads to loss of high-affinity binding. *J. Biol. Chem.* **265**: 9595-9598.

Hempstead, B.L., Rabin, S.J., Kaplan, L., Reid, S., Parada, L.F., Kaplan, D.R. (1992). Overexpression of the *trk* tyrosine kinase rapidly accelerates nerve growth factor-induced differentiation. *Neuron* **9**: 883-896.

Hempstead, B.L., Schleifer, L.S., Chao, M.V. (1989). Expression of functional NGF receptors after gene transfer. *Science* **243**: 373-376.

Henderson, C.E., Camu, W., Mettling, G.,Gouin, A., Poulsen, K., Karaloo, M., Rullamas, J., Evans, T., McMahon, S.B., Armanini, M.P., Berkemeier, L., Phillips, H.S., Rosenthal, A. (1993). Neurotrophins promote motoneuron survival and are present in embryonic limb bud. *Nature* **363**: 266-270.

Henderson, C.E., Phillips, S.H., Pollock, R.A., Davies, A.M., Lemeulle, C., Armanini, M., Simpson, L.C., Moffet, B., Vandlen, R.A., Koliatsos, V.E., Rosenthal, A. (1994). GDNF: a potent survival factor for motoneurons present

in peripheral nerve and muscle. *Science* **266**: 1062-1064.

Heumann, R. (1994). Neurotrophin signalling. *Curr. Opin. Neurobiol.* **4**: 668-679.

Heumann, R., Korsching, S., Scott, J., and Thoenen, H. (1984). Relationship between levels of nerve growth factor (NGF) and its messenger RNA in sympathetic ganglia and peripheral target tissues. *EMBO J.* **3**: 3183-3189.

Heumann, R., Lindholm, D., Bandtlow, C., Meyer, M., Radeke, M.J., Misko, T.P., Shooter, E, Thoenen, H. (1987). Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerve during development, degeneration, and regeneration: role of macrophages. *Proc. Natl Acad. Sci. USA* **84**: 8735-8739.

Higgins, G.A., Koh, S., Chen, K.S., Gaga, F.H. (1989). NGF induction of NGF receptor gene expression and cholinergic neuronal hypertrophy within the basal forebrain of the adult rat. *Neuron* **3** 247-256.

Hirata, T., Morii, E., Morimoto, M., Kasugai, T., Tsujimura, T., Hirota, S., Kanakura, Y., Nomura, S., Kitamura, Y. (1993). Stem cell factor induces outgrowth of *c-kit*-positive neurites and supports the survival of *c-kit*-positive neurons in dorsal root ganglia of mouse embryos. *Development* **119**: 49-56.

Hiscock, J., Straznicky, C. (1986). The development of the neurons of the glossopharyngeal (IX) and vagal (X) sensory ganglia in chick embryos. *Histol. Histopath.* 1: 129-137.

Hofer, M.M., Barde, Y-A. (1988). Brain-derived neurotrophic factor prevents neuronal death *in vivo*. *Nature* **331**: 261-262.

Hoffman, H-D., Unsicker, K. (1982). The seminal vesicle of the bull: New and very rich source of nerve growth factor. *Eur. J. Biochem.* **128**: 421-426.

Hohn, A., Leibrock, J., Bailey, K., Barde, Y-A. (1990). Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. *Nature* **344**: 339-341.

Honig, M.G., Hume, R.I. (1986). Fluorescent carbocyanine dyes allow living neurons of identified origin to be studied in long-term cultures. *J. Cell Biol.* **103**: 171-

197.

Hory-Lee, F., Russell, M., Lindsay, R.M., Frank, E. (1993). Neurotrophin-3 supports the survival of developing muscle sensory neurons in culture. *Proc. Natl. Acad. Sci. USA* **90**: 2613-2617.

Houenou, L., Prevette, D., and Oppenheim, R. (1989). Motoneuron survival *in vivo* following treatment with extracts from active and inactive muscle. *Soc. Neurosci. Abstr.* **15**: 436.

Hughes, R.A., Sendtner, M., Goldfarb, M., Lindholm, D., Thoenen, H. (1993). Evidence that fibroblast growth factor 5 is a muscle-derived survival factor for cultured spinal motoneurons. *Neuron* **10**: 369-377.

Hyman, C., Hofer, M., Barde, Y-A., Juhasz, M., Yancopoulos, G.D., Squinto, S.P., Lindsay, R.M. (1991). BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. *Nature* **350**: 230-232.

Ibáñez, C.F., Ebandal, T., Barbany, G., Murray-Rust, J., Blundell, T.L., Persson, H. (1992). Disruption of the low-affinity receptor binding site in NGF allows neuronal survival and differentiation by binding to the product of the *trk* gene. *Cell* **69**: 329-341.

Ip, N.Y., Ibáñez, C.F., Nye, S.H., McClain, J., Jones, P.F., Gies, D.R., Belluscio, L., Le Beau, M.M., Espinosa III, R., Squinto, S.P., Persson, H., Yancopoulos, G.D. (1992). Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. *Proc. Natl Acad. Sci. USA* **89**: 3060-3064.

Ip, N.Y., Li, Y.P., Ivan, D.S., Panayotatos, N., Alderson, R.F., Lindsay, R.M. (1991). Ciliary neurotrophic factor enhances neuronal survival in embryonic rat hippocampal cultures. *J. Neurosci.* **11**: 3124-3134.

Ip, N.Y., McClain, J., Barrezueta, N.X., Aldrich, T.H., Pan, L., Li, Y., Wiegand, S.J., Friedman, B., Davis, S., Yancopoulos, G.D. (1993a). The alpha component of the CNTF receptor is required for for signalling and defines potential CNTF targets in the adult and during development. *Neuron* **10**: 89-

102.

Ip, N.Y., Stitt, T.N., Tapley, P., Klein, R., Glass, D.J., Fandl, J., Greene, L.A., Barbacid, M., Yancopoulos, G.D. (1993b). Similarities and differences in the way neurotrophins interact with the *trk* receptors in neuronal and nonneuronal cells. *Neuron* **10**: 137-149.

Isackson, P.J., Huntsman, M.M, Murray, K.D., Gall, C.M. (!991). BDNF mRNA expression is increased in adult rat forebrain after limbic seizures: temporal patterns of induction distinct from NGF. *Neuron* **6**: 937-948.

Jing, S., Tapley, P., Barbacid, M. (1992). Nerve growth factor mediates signal transduction through trk homodimer receptors. *Neuron* **9**: 1067-1079.

Johnson, E.M., Gorin, P.D., Brandeis, L.D., Pearson, J. (1980). Dorsal root ganglion neurons are destroyed by *in utero* exposure to maternal antibody to nerve growth factor. *Science* **210**: 926-918.

Jones, K.R., Fariñas, I., Backus, C., Reichardt, L.F. (1994). Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. *Cell* **76**: 989-1000.

Kalcheim, C., Barde, Y-A., Thoenen, H., Le Douarin, N. (1987). *In vivo* effect of brain-derived neurotrophic factor on the survival of developing dorsal root ganglion cells. *EMBO J.* **6**: 2871-2873.

Kalcheim, C., Carmeli, C., Rosenthal, A. (1992). Neurotrophin-3 is a mitogen for cultured neural crest cells. *Proc. Natl. Acad. Sci. USA* **89**: 1661-1665.

Kalcheim, C., Gendreau, M. (1988). Brain-derived neurotrophic factor stimulates survival and neuronal differentiation in cultured avian neural crest. *Brain Res.* **469**: 76-86.

Kapeller, R., Chakrabarti, R., Cantley, L., Fay, F., Corvera, S. (1993). Internalization of activated platelet-derived growth factor receptor-phosphatidylinositol-3' kinase complexes: potential interactions with the microtubule cytoskeleton. *Mol. Cell. Biol.* **13**: 6052-6063.

Kaplan, D.R., Hempstead, B.L., Martin-Zanca, D., Chao, M.V., Parada, L.F.

(1991a). The *trk* proto-oncogene product: a signal transducing receptor for nerve growth factor. *Science* **252**: 554-558.

Kaplan, D.R., Martin-Zanca, D., Parada, L.F. (1991b). Tyrosine phosphorylation and tyrosine kinase activity of the *trk* proto-oncogene product is induced by NGF. *Nature* **350**: 158-160.

Kaplan, D.R., Stephens, R.M. (1994). Neurotrophin signal transduction by the *trk* receptor. *J. Neurobiol.* **25**: 1404-1417.

Keshet, E., Lyman, S.D., Williams, D.E., Anderson, D.M., Jenkins, N.A., Copeland, N.G., Parada, L.F. (1991). Embryonic RNA expression patterns of the *c-kit* receptor and its cognate ligand suggest multiple functional roles in mouse development. *EMBO J.* **10**: 2425-2235.

Klein, R., Conway, D., Parada, L.F., Barbacid, M. (1990a). The *trk*B tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. *Cell* **61**: 647-656.

Klein, R., Jing, S., Nanduri, V., O'Rourke, E., Barbacid, M. (1991a). The *trk* proto-oncogene encodes a receptor for nerve growth factor. *Cell* **65**: 189-197.

Klein, R., Lamballe, F., Bryant, S., Barbacid, M. (1992). The *trk*B tyrosine protein kinase is a receptor for neurotrophin-4. *Neuron* **8**: 947-956.

Klein, R., Martin-Zanca, D., Barbacid, M., Parada, L.F. (1990b). Expression of the tyrosine kinase receptor gene *trk*B is confined to the murine embryonic and adult nervous system. *Development* **109**: 845-850.

Klein, R., Nanduri, V., Jing, S., Lamballe, F., Tapley, P., Bryant, S., Cordon-Cardo, C., Jones, K.R., Reichardt, L.F., Barbacid, M. (1991b). The *trk*B tyrosine kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. *Cell* **66**: 395-403.

Klein, R., Silos-Santiago, I., Smeyne, R.J., Lira, S.A., Brambrilla, R., Bryant, S., Zhang, L., Snider, W.D., Barbacid, M. (1994). Targeted disruption of *trk*C, the neurotrophin-3 receptor gene, eliminates 1a muscle afferents and results in loss of proprioception. *Nature* **386**: 249-251.

Klein, R., Smeyne, R.J., Wurst, W., Long, L.K., Auerbach, B.A., Joyner A.L., Barbacid, M. (1993). Targeted disruption of the *trk*B neurotrophin receptor gene results in nervous sytem lesions and neonatal death. *Cell* **75**: 113-122.

Koliatsos, V.E., Clatterbuck, R.E., Winslow, J.W., Cayouette, M.H., Price, D.L. (1993). Evidence that brian-derived neurotrophic factor is a trophic factor for motor neurons *in vivo*. *Neuron* **10**: 359-367.

Korsching, S. (1993). The neurotrophic factor concept: a reexamination. *J. Neurosci.* **13**: 2739-2748.

Krust, A., Kastner, P., Petkovich, M., Zelent, A., Chambon, P. (1989). A third human retinoic acid receptor, hRAR-γ. *Proc. Natl Acad. Sci. USA* **86** :5310-5314.

Lamballe, F., Klein, R., Barbacid, M. (1991). *trk*C, a new member of the *trk* family of tyrosine protein kinases, is a receptor for neurotrophin-3. *Cell* **66**: 967-979.

Lamballe, F., Smeyne, R.J., Barbacid, M. (1994). Developmental expression of *trk*C, the neurotrophin-3 receptor, in the mammalian nervous system. *J. Neurosci.* **14**: 14-28.

Large, T.H., Bodary, S.C., Clegg, D.O., Weskamp, G., Otten, U., Reichardt, L.F. (1986). Nerve growth factor gene expression in the developing rat brain. *Science* **234**: 352-354.

Large, T.H., Weskamp, G., Helder, J.C., Radeke, M.J., Misko, T.M., Shooter, E.M., and Reichardt, L.F. (1989). Structure and developmental expression of the nerve growth factor receptor in the chicken central nervous system. *Neuron* **2**: 1123-1134.

Larmet, Y., Dolphin, A.C., Davies, A.M. (1992). Intracellular calcium regulates the survival of early sensory neurons before they become dependent on neurotrophic factors. *Neuron* **9**: 563-574.

Lebeau, M., Alveras-Bolado, G., Braissant, O., Wahli, W., and Catsicas, S. (1991). Ribosomal protein L27 is identical in the chick and rat . *Nucleic Acids Res.* **19**: 1337.

Lee, K-F., Bachman, K., Landis, S., Jaenisch, R. (1994a). Dependence on p75 for innervation of some sympathetic targets. *Science* **263**: 1447-1449.

Lee, K-F., Davies, A.M., Jaenisch, R. (1994b). p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF. *Development* **120**: 1027-1033.

Lee, K-F., Li, E., Huber, L. J., Landis, S.C., Sharpe, A.H., Chao, M.V., Jaenisch, R. (1992). Targeted mutation of the gene encoding the low-affinity NGF receptor p75 leads to deficits in the peripheral nervous system. *Cell* **69**: 737-749.

Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P., Thoenen, H., Barde, Y-A. (1989). Molecular cloning and expression of brain-derived neurotrophic factor. *Nature* **34**: 149-152.

Levi-Montalcini, R. (1987). The nerve growth factor 35 years later. *Science* **237**: 1154-1162.

Levi-Montalcini, R., Angeletti, P. (1968). Nerve growth factor. *Physiol. Rev.* **48**: 534-569.

Levi-Montalcini, R., Hamburger, V. (1951). Selective growth-stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *J. Exp. Zool.* **116**: 321-361.

Levi-Montalcini, R., Hamburger, V. (1953). A diffusible agent of mouse sarcoma, producing hyperplasia of sympathetic ganglia and hyperneurotization of viscera in the chick embryo. *J. Exp. Zool.* **123**: 233-287.

Levi-Montalcini, R., Meyer, H., Hamburger, V. (1954). *In vitro* experiments on the effects of mouse sarcomas 180 and 37 on the spinal and sympathetic ganglia of the chick embryo. *Cancer Res.* **14**: 49-57.

Li, B-Q., Kaplan, D., Kung, H., Kamata, T. (1992). Nerve growth factor stimulation of Ras-guanine nucleotide exchange factor and Gap activities. *Science* **256**: 1456-1459.

Lin, L-F., Armes, L.G., Sommer, A., Smith, D.J., Collins, F. (1990). Isolation and

characterization of ciliary neurotrophic factor from rabbit sciatic nerves. *J. Biol. Chem.* **265**: 8942-8947.

Lin, L-F. H., Doherty, D.H., Lile, J.D., Bektesh, S., Collins, F. (1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260**: 1130-1132.

Lindholm, D., Hengere, B., Zafra, F., Thoenen, H. (1990). Transforming growth factor β1 stimulates expression of nerve growth factor in rat CNS. *NeuroReport* **1**: 9-12.

Lindsay, R.M. (1988). Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. *J. Neurosci.* **8**: 2394-2405.

Lindsay, R.M., Shooter, E.M., Radeke, M.J., Misko, T.P., Déchant, G., Thoenen, H., Lindholm, D. (1990). Nerve growth factor regulates expression of the nerve growth factor receptor in adult sensory neurons. *Eur. J. Neurosci.* **2**: 389-396.

Lindsay, R.M., Thoenen, H., Barde, Y-A. (1985). Placode- and neural crest-derived sensory neurons are responsive at early developmental stages to brain-derived neurotrophic factor. *Dev. Biol.* **112**: 319-328.

Lipton, S.A., Kater, S.B. (1989). Neurotransmitter regulation of neuronal outgrowth, plasticity and survival. *Trends Neurosci.* **12**: 265-270.

Lipton, S.A., Wagner, J.A., Madison, R.D., D'Amore, P.A. (1988). Acidic fibroblast growth factor enhances regeneration of processes by postnatal retinal ganglion cells in culture. *Proc. Natl Acad. Sci. USA* **85**: 2388-2392.

Loeb, D.M., Tsao, H., Cobb, M.H., Greene, L.A. (1992). NGF and other growth factors induce an association between ERK 1 and the NGF receptor, gp140^{prototrk}. *Neuron* **9**: 1053-1065.

Lu, B., Buck, C.R., Dreyfus, C.F., Black, I.B. (1989). Expression of NGF and NGF receptor mRNA in the developing brain: evidence for local delivery and action of NGF. *Exp. Neurol.* **104**: 191-199.

Maisonpierre, P.C., Belluscio, L., Conover, J.C., Yancopoulos, G.D. (1992). Gene sequences of chicken BDNF and NT-3. *DNA Sequence* **3**: 49-54.

Maisonpierre, P.C., Belluscio, L., Squinto, S., Ip, N.Y., Furth, M.E., Lindsay, R.M., Yancopoulos, G.D. (1990). Neurotrophin-3: a neurotrophin related to NGF and BDNF. *Science* **247**: 1446-1451.

Maisonpierre, P.C., Le Beau, M.M., Espinosa III, R., Ip, N.Y., Belluscio, L., De La Monte, M., Squinto, S., Furth, M.E., Yancopoulos, G.D. (1991). Human and rat brain-dreived neurotrophic factor and neurotrophin-3: gene structures, distributions and chromosomal localizations. *Genomics* **10**: 558-568.

Manthorpe, M., Skaper, S.D., Williams, L.R., Varon, S. (1986). Purification of adult rat sciatic nerve ciliary neurotrophic factor. *Brain Res.* **367**: 282-286.

Martin-Zanca, D., Barbacid, M., Parada, L.F. (1990). Expression of the *trk* proto-oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development. *Genes Dev.* **4**: 683-694.

Martin-Zanca, D., Oskam, R., Mitra, G., Copeland, T., Barbacid, M. (1989). Molecular and biochemical characterization of the human *trk* proto-oncogene. *Mol. Cell Biol* **9**: 24-33.

Masiakowski, P., Liu, H., Radziejewski, C., Lottspeich, F., Oberthuer, W., Wong, V., Lindsay, R.M., Furth, M.E., Panayotatos, N. (1991). Recombinant human and rat ciliary neurotrophic factors. *J. Neurochem.* **57**: 1003-1012.

Massagué, J., Attisano, L., Wrana, J.L. (1994). The TGF-β family and its composite receptors. *Trends Cell Biol.* **4**: 172-177.

Masu, Y., Wolf, E., Holtmann, B., Sendtner, M., Brem, G., Thoenen, H. (1993). Disruption of the CNTF gene results in motor neuron degeneration. *Nature* **365**: 27-32.

Matsui, Y., Zsebo, K.M., Hogan, B.L.M. (1990). Embryonic expression of a haematopoietic growth factor encoded by the *Sl* locus and the ligand for c-kit. *Nature* **347**: 667-669.

Matsushima, H., Bogenmann, E. (1990). Nerve growth factor induces neuronal

differentiation in neuroblastoma cells transfected with the NGF receptor cDNA. *Mol. Cell Biol.* **10**: 5015-5020.

McCormick, F. (1994). Activators and effectors of ras p21 proteins. *Curr. Opin. Genet. Dev.* **4**: 71-76.

McDonald, N.Q., Lappatto, R. Murry-Rust, J., Gunning, J., Wlodawer, A., Blundell, T.L. (1991). New protein fold revealed by a 2.3-Å resolution crystal structure of nerve growth factor. *Nature* **354**: 411-414.

Meakin, S.O., Shooter, E.M. (1992). The nerve growth factor family of receptors. *Trends Neurosci.* **15**: 323-331.

Meakin, S.O., Suter, U., Drinkwater, C.C., Welcher, A.A., Shooter, E.M. (1992). The rat *trk* proto-oncogene product exhibits properties characteristic of the slow nerve growth factor receptor. *Proc. Natl. Acad. Sci. USA* **89**: 2374-2378.

Merlio, J.P., Ernfors. P., Jaber, M., Persson, H. (1992). Molecular cloning of rat *trk*C and distribution of cells expressing messenger RNAs for members of the *trk* family in the rat central nervous system. *Neuroscience* **51**: 513-532.

Middlemas, D.S., Lindberg, R.A., Hunter, T. (1991). *trk*B, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. *Mol. Cell. Biol.* **11**: 143-153.

Miller, F.D., Mathew, T.C., Toma, J.G. (1991). Regulation of nerve growth factor receptor gene expression in the peripheral nervous system. *J. Cell Biol.* **112**: 303-312.

Mobley, W.C., Woo, J.E., Edwards, R.H., Riopelle, R.J., Longo, F.M., Weskamp, G., Otten, U., Valletta, J.S., Johnston, M.V. (1989). Developmental regulation of nerve growth factor and its receptor in the rat caudate-putamen. *Neuron* **3**: 655-664.

Montero, C.N., Reid, K., Brown, M.A., Bartlet, P.F. (1988). Rescue of lesioned septal cholinergic neurons by nerve growth factor: specificity and requirement for chronic treatment. *J. Neurosci.* **8**: 2986-2999.

Motro, B., Van der Kooy, D., Rossant, J., Reith, A., Bernstein, A. (1991).

Contiguous patterns of *c-kit* and *steel* expression: analysis of mutations at the *W* and *Sl* loci. *Development* **113**: 1207-1221.

Murphy, R.A., Chlumecky, V., Smillie, L.B., Carpenter, M., Nattris, M., Anderson, J.K., Rhodes, J.A., Barker, P.A., Siminoski, K., Campenot, R.B., Haskins, J. (1989). Isolation and characterisation of a glycosylated form of beta-nerve growth factor in mouse submandibular glands. *J. Biol. Chem.* **264**: 12502-12509.

Neufeld, G., Gospodarowicz, D., Dodge, L., Fujii, D.K. (1987). Heparin modulation of the neurotrophic effects of acidic and basic fibroblast growth factors and nerve growth factor on PC12 cells. *J. Cell Physiol.* **131**: 131-140.

Nichols, N.R., Laping, N.J., Day J.R., Finch, C.E. (1991). Increases in transforming growth factor-β mRNA in hippocampus during response to entorhinal cortex lesions in intact an adrenalectomized rats. *J. Neurosci. Res.* **28**: 134-139.

Nishi, R., Berg, D.K. (1981). Effects of high K^+ concentrations on the growth and development of ciliary ganglion neurons in cell culture. *Dev. Biol.* **87**: 301-307.

Okado, N., Oppenheim, R.W. (1984). Cell death of motoneurons in the chick embryo spinal cord. IX. The loss of motoneurons following removal of afferent inputs. *J. Neurosci.* **4**: 1639-1652.

Oppenheim, R.W. (1989). The neurotrophic theory and naturally occurring motoneuron death. *Trends Neurosci.* **12**: 252-255.

Oppenheim, R.W. (1991). Cell death during development of the nervous system. *Ann. Rev. Neurosci.* **14**: 453-501.

Oppenheim, R.W., Houenou, L.J., Johnson, J.E., Lin, L-F.H., Li, Linxi, Lo, A.C., Newsome, A.L., Prevette, D.M., Wang, S. (1995). Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. *Nature* **373**: 344-346.

Oppenheim, R.W., Prevette, D.M., Tytell, D., Homma, S. (1991). Naturally ocurring and induced neuronal death *in vivo* requires protein and RNA synthesis:

Evidence for the role of cell death genes. *Devel. Biol.* **138**: 104-113.

Oppenheim, R.W., Qin-Wei, Y., Prevette, D., Yan, Q. (1992). Brain-derived neurotrophic factor rescues developing avian motoneurons from cell death. *Nature* **360**: 755-757.

Persson, H., Ernfors, P. 1992. Visualization and quantification of neurotrophin mRNAs. *Neuromethods, Volume 27. Neurotrophic Factors.* Academic Press.

Pirvola, U., Ylikoski, J., Palgi, J., Lehtonen, E., Arumae, U., Saarma, M. (1992). Brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the peripheral target fields of developing inner ear ganglia. *Proc. Natl Acad. Sci. USA* **89**: 9915-9919.

Pleasure, S.J., Reddy, U.R., Venkatakrishnan, G., Roy, A.K., Chen, J., Ross, A.H., Trojanowski, J.Q., pleasure, D.E., Lee, V.M.Y. (1990). Introduction of NGF receptors into medulloblastoma cell line results in expression of high- and low-affinity receptors but not NGF-mediated differentiation. *Proc. Natl Acad. Sci. USA* **87**: 8496-8500.

Phillips, H.S., Hains, J.M., Laramee, G.R., Rosenthal, A., Winslow, J.W. (1990). Widespread expression of BDNF but not NT-3 by target areas of basal forebrain cholinergic neurons. *Science* **250**: 290-294.

Rabin, S.J., Cleghorn, V., Kaplan D.R. (1993). SNT, a differentiation specific target of neurotrophic factor-induced tyrosine kinase activity in neurons and PC12 cells. *Mol. Cell. Biol.* **13**: 2203-2213.

Radeke, M.J., Misko, T.P., Hsu, C., Herzenberg, L.A., Shooter, E.M. (1987). Gene transfer and molecular cloning of the rat nerve growth factor receptor. *Neuron* **4**: 487-492.

Rhee, S.G., Choi, K.D. (1992). Regulation of inositol phospholipid-specific phospholipase C isozymes. *J. Biol. Chem.* **267**: 12393-12396.

Rodriguez-Tébar, A., Déchant, G., Barde, Y-A. (1990). Binding of neurotrophin-3 to its neuronal receptors and its interactions with nerve growth factor and brain-derived neurotrophic factor. *EMBO J.* **11**: 917-922.

Rodriguez-Tébar, A., Rohrer, H. (1991). Retinoic acid induces NGF-dependent survival response and high-affinity NGF receptors in immature chick sympathetic neurons. *Development* **112**: 813-820.

Rogers, L.A., Cowan, W.M. (1974). The development of the mesencephalic nucleus of the trigeminal nerve in the chick. *J. Comp. Neurol.* **147**: 291-320.

Rosenthal, A., Goeddel, D.V., Nguyen, T., Lewis, M., Shih, A., Laramee, G.R., Nikolics, K., Winslow, J.W. (1990). Primary structure and biological activity of a novel human neurotrophic factor. *Neuron* **4**: 767-773.

Rowe, A., Eager, N.S.C., Brickell, P.M. (1991). A member of the RXR nuclear receptor family is expressed in neural crest-derived cells in the developing chick peripheral nervous system. *Development* **111**: 771-778.

Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P.G., Schlessinger, J., Pawson, T. (1992). Association of the Shc and Grb2/Sem 5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature* **360**: 689-692.

Ruit, K.G., Elliott, J.L., Osborne, P.A., Yan, Q., Snider, W.D. (1992). Selective dependence of mammalian dorsal root ganglion neurons on nerve growth factor during embryonic development. *Neuron* **8**: 572-587.

Rudge, J.S., Li, Y., Pasnikowski, E.M., Mattsson, K., Pan, L., Yancopoulos, G.D., Weigand, S.J., Lindsay, R.M., Ip, N.Y. (1994). Neurotrophic factor receptors and their signal transduction capabilites in rat astrocytes. *Eur. J. Neurosci.* **6**: 693-705.

Saadat, S., Sendtner, M., Rohrer, H. (1989). Ciliary neurotrophic factor induces cholinergic differentiation of rat sympathetic neurons in culture. *J. Cell Biol.* **108**: 1807-1816.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning: A laboratory manual 2nd. ed. Vols 1, 2, and 3. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Scarpini, E. Ross, A.H., Rosen, J.L., Brown, M.J., Rostami, A., Koprowski, H., Lisak, R.P. (1988). Expression of nerve growth factor receptor during human peripheral nerve development. *Devel. Biol.* **125**: 301-310.

Schechter, A.L., Bothwell, M.A. (1981). Nerve growth factor receptors on PC12 cells: evidence for two receptor classes with differing cytoskeletal association. *Cell* **24**: 867-874.

Schecterson, L.C., Bothwell, M. (1992). Novel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons. *Neuron* **9**: 449-463.

Schecterson, L.C., Bothwell, M. (1994). Neurotrophin and neurotrophin receptor mRNA expression in developing inner ear. *Hear. Res.* **73**: 92-100.

Scheibe, R.J., Wagner, J.A. (1992). Retinoic acid regulates both expression of the nerve growth factor receptor and sensitivity to nerve growth factor. *J. Biol. Chem.* **267**: 17611-17616.

Schoenwolf, G.C., Smith, J.L. (1990). Mechanisms of neurulation: traditional viewpoint and recent advances. *Development* **109**: 243-270.

Selby, M.J., Edwards, R., Harp, F., Rutter, W.J. (1987). Mouse nerve growth factor gene: Structure and expression. *Mol. Cell. Biol.* **7**: 3057-3064.

Shelton, D.L. and Reichardt, L.F. (1984). Expression of the beta-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. *Proc. Natl Acad. Sci. USA* **81**: 7951-7955.

Shelton, D.L., Sutherland, J., Gripp, J., Camerato, T., Armanini, M.P., Philips, H.S., Carroll, K., Spencer, S.D., Levinson, A.D. (1995). Human trks: Molecular cloning, tissue distribution, and expression of extracellular domain immunoadhesins. *J. Neurosci.* **15**:477-491.

Shoelson, S.E., Sivaraja, M., Williams, K.P., Hu., P., Schlessinger, J., Weiss, M.A. (1993). Specific phosphopeptide binding regulates a conformational change in the PI-3 kinase SH2 domain associated with enzyme activation. *EMBO J.* **12**: 795-802.

Sidell, N. (1982). Retinoic acid-induced growth inhibition and morphologic differentiation of human neuroblastoma cells *in vitro*. *J. Nat. Cancer Inst.* **68**: 589-596.

Sieber-Blum, M. (1991). Role of neurotrophic factors BDNF and NGF in the commitment of pluripotent neural crest cells. *Neuron* **6**: 949-955.

Smeyne, R.J, Klein, R., Schnapp, A., Long, L.K., Bryant, S., Lewin, A., Lira, S.A., Barbacid, M. (1994). Severe sensory and sympathetic neuropathies in mice carrying a disrupted *trk*/NGF receptor gene. *Nature* **368**: 246-249.

Snider, W.D. (1994). Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. *Cell* **77**: 627-638.

Songyang, Z., Shoelson, S.E., Chauduri, M., Gish, G, Pawson, T., Haser, W.G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R.J., Neel, B.G., Birge, R.B., Fajardo, J.E., Chou, M.M., Hanafusa, H., Schauffhausen, B., Cantley, L.C. (1993). SH2 domains recognize specific phosphopeptide sequences. *Cell* **72**: 767-778.

Soppet, D., Escandon, E., Maragos, J., Middlemas, D.S., Reid, S.W., Blair, J., Burton, L.E., Stanton, B.R., Kaplan, D.R., Hunter, T., Nikolics K., Parada,. L.F. (1991). The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the *trk*B tyrosine kinase receptor. *Cell* **65**: 895-903.

Squinto, S.P., Stitt, T.N. Aldrich, T.H., Davis, S., Bianco, S.M., Radiejewski, C., Glass, D.J., Masiakowski, P., Furth, M.E., Valenzuela, D.M., Distefano, P.S., Yancopoulos, G.P. (1991). *trk*B encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. *Cell* **65**: 885-893.

Steininger, T.L., Wainer, B.H., Klein, R., Barbacid, M., Palfrey, H.C. (1993). High-affinity nerve growth factor receptor (*trk*) immunoreactivity is localized in cholinergic neurons of the basal forebrain and striatum in the adult rat brain. *Brain Res.* **612**: 330-335.

Stephens, R.M., Loeb, D.M., Copeland, T.D., Pawson T., Greene, L.A., Kaplan, D.R. (1994). Trk receptors use redundant signal transduction pathways involving SHC and PLC-γ1 to mediate NGF responses. *Neuron* **12**: 691-705.

Stöckli, K.A., Lottspeich, F., Sendtner, M., Masiakowski, P., Carroll, P., Götz, R., Lindholm, D., Thoenen, H. (1989). Molecular cloning, expression and regional distribution of rat ciliary neurotrophic factor. *Nature* **342**: 920-923.

Suen, K-L., Bustelo, X.R., Pawson, T., Barbacid, M. (1993). Molecular cloning of the mouse *grb2* gene: differential interaction of the Grb2 adaptor protein with epidermal growth factor and nerve growth factor receptors. *Mol. Cell. Biol.* **13**: 5500-5512.

Sutter, A., Riopelle, R.J., Harris-Warrick, R.M., Shooter, E.M. (1979). NGF receptors: characterization of two distinct classes of binding sites on chick embryo sensory ganglia cells. *J. Biol. Chem.* **254**: 5972-5982.

Tanaka, H. (1987). Chronic application of curare does not increase the level of motoneuron survival-promoting activity in limb muscle extracts during naturally occurring motoneuron cell death period. *Dev. Biol.* **124**: 347-357.

Tessarollo, L., Tsoulfas, P., Martin-Zanca, D., Gilbert, D.J., Jenkins, N.A., Copeland, N.G., Parada, L.F. (1993). *trk*C, a receptor for neurotrophin-3, is widely expressed in the developing nervous system and in non-neuronal tissues. *Development* **118**: 463-475.

Thoenen, H. (1991). The changing scene of neurotrophic factors. *Trends Neurosci.* **14**: 165-169.

Thoenen, H., Bandtlow, C., Heumann, R. (1987). The physiological function of nerve growth factor in the central nervous system: comparison with the periphery. *Rev. Physiol. Biochem. Pharmacol.* **109**: 145-178.

Thoenen, H., Barde, Y-A. (1980). Physiology of nerve growth factor. *Physiol. Rev.* **60**: 1284-1335

Timmusk, T., Palm, K., Metsis, M., Reintam, T., Paalme, V., Saarma, M., Persson, H. (1993). Multiple promoters direct tissue-specific expression of the rat BDNF

gene. *Neuron* **10**: 475-489.

Tocco, M.D., Contreras, S., Koizumi, S., Dickens, G., Guroff, G. (1988). Decreased levels of nerve growth factor receptor on dexamethasone-treated PC12 cells. *J. Neurosci. Res.* **20**: 411-416.

Tomac, A., Lindqvist, E., Lin, L-F., H., Ögren, S.O., Young, D., Hoffer, B.J., Olson L. (1995). Protection and repair of the nigrostriatal dopaminergic system by GDNF *in vivo*. *Nature* **373**: 335-339.

Unsicker, K., Reichert-Preibsch, H., Schmidt, R., Pettman, B., Labourdette, G., Sensenbrenner, M. (1987). Astroglial and fibroblast growth factors have neurotrophic functions for cultured peripheral and central nervous system neurons. *Proc. Natl Acad. Sci. USA* **84**: 5459-5463.

Valenzuela, D.M., Maisonpierre, P.C., Glass, D.J., Rojas, E., Nuñez, L., Kong, Y., Gies, D.R., Stitt, T.N., Ip, N.Y., Yancopoulos, G.D. (1993). Alternative forms of rat *trk*C with different functional capabilities *Neuron* **10**: 963-974.

Vantini, G., Schiavo, N., Di., M.A., Polato, P., Triban, C., Callegaro, L., Toffano, G., Leon, A. (1989). Evidence for a physiological role of nerve growth factor in the central nervous system of neonatal rats. *Neuron* **3**: 267-273.

Verge, V.M.K., Merlio, J., Grondin, J., Ernfors, P., Persson, H., Riopelle, R.J., Hokfelt, T., Richardson, P.M. (1992). Co-localization of NGF binding sites, *trk* mRNA, and low-affinity NGF receptor mRNA in primary sensory neurons: Responses to injury and infusion of NGF. *J. Neurosci.* **12**: 4011-4022.

Vogel, K.S., Davies, A.M. (1991). The duration of neurotrophic factor independence in early sensory neurons is matched to the time course of target field innervation. *Neuron* **7**: 819-830.

von Bartheld, C.S., Patterson, S.L., Heuer, J.G., Wheeler. E.F., Bothwell, M., Rubel, E.W. (1991). Expression of nerve growth factor (NGF) receptors in the developing inner ear of chick and rat. *Development* **113**: 455-470.

Walicke, P.A. (1988). Basic and acidic fibroblast growth factors have trophic effects on neurons from multiple CNS regions. *J. Neurosci.* **8**: 2618-2627.

Walicke, P., Cowan, W.M., Ueno, N., Baird, A., Guillemin, R. (1986). Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. *Proc. Natl Acad. Sci. USA* **83**: 3012-3016.

Welcher, A.A., Bitler, C.M., Radeke, M., Shooter, E.M. (1991). Nerve growth factor binding domain of the nerve growth factor receptor. *Proc. Natl. Acad. Sci. USA* **88**: 159-163.

Weskamp, G., Reichardt, L.F. (1991). Evidence that biological activity of NGF is mediated through a novel subclass of high affinity receptors. *Neuron* **6**: 139-150.

Wetmore, C., Cao, Y., Patterson, R.F., Olsen, L. (1991). Brain-derived neurotrophic factor: subcellular compartmentalization and interneuronal transfer as visualized with anti-peptide antibodies. *Proc. Natl Acad. Sci. USA* **88**: 9843-9847.

Wheeler, E.F., Bothwell, M. (1992). Spatiotemporal patterns of expression of NGF and the low-affinity NGF receptor in rat embryos suggest functional roles in tissue morphogenesis and myogenesis. *J. Neurosci.* **12**: 930-945.

Whitman, M., Kaplan, D., Roberts, T., Cantley, L. (1987). Evidence for two distinct phosphatidylinositol kinases in fibroblasts: implications for cellular regulation. *Biochem. J.* **247**: 165-174.

Wright, E.M., Vogel, K.S., Davies, A.M. (1992). Neurotrophic factors promote the maturation of developing sensory neurons before they become dependent on these factors for survival. *Neuron* **9**: 139-150.

Wyatt, S., Davies, A.M. (1993). Regulation of expression of mRNAs encoding the nerve growth factor receptors p75 and *trk*A in developing sensory neurons. *Development* **119**: 635-647.

Wyatt, S., Davies, A.M (1995). Regulation of expression of p75 and *trk*A mRNAs in developing sympathetic neurons. *J. Cell Biol.* **130**: 1435-1446.

Wyatt, S., Shooter, E.M., Davies, A.M. (1990). Expression of the nerve growth receptor gene in sensory neurons and their cutaneous targets prior to and during innervation. *Neuron* 4:421-427.

Yan H., Schlessinger, J., Chao, M.V. (1991). Chimeric NGF-EGF receptors define domains responsible for neuronal differentiation. *Science* **252**: 561-563.

Yan, Q., Johnson, E.J. (1988). An immunohistochemical study of the nerve growth factor receptor in developing rats. *J. Neurosci.* **8**: 3481-3498.

Yan, Q., Matheson, C., Lopez, O.T. (1995). *In vivo* neurotrophic effects of GDNF on neonatal and adult facial motor neurons. *Nature* **373**: 341-344.

Zhan, X., Bates, B., Hu, X., Goldfarb, M. (1988). The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors. *Mol. Cell. Biol.* **8**: 3487-3495.

Zhan, X., Culpepper, A., Reddy, M., Loveless, J., Goldfarb, M. (1987). Human oncogenes detected by a defined medium culture assay. *Oncogene* **1**: 369-376.

Zhang, D., Yao, L., Bernd, P. (1994). Expression of *trk* and neurotrophin mRNA in dorsal root and sympathetic ganglia of the quail during development. *J. Neurobiol.* **25**: 1517-1532.